University of Alberta

Evolutionary and Nutritional Importance of Phospholipid Methylation

by

Christopher J. Walkey

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Department of Biochemistry

Edmonton, Alberta

Spring 1998

National Library of Canada
Acquisitions and Bibliographic Services
395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque nationale du Canada
Acquisitions et services bibliographiques
395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence

Our file Notre référence

The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

L'auteur conserve la propriété du droit d'auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

0-612-29121-9

Canada

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Evolutionary and Nutritional Importance of Phospholipid Methylation submitted by Christopher J. Walkey in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

D. E. Vance
Dennis Vance, Supervisor

Susan A. Henry
Susan Henry, External Examiner

Luis Agellon

James C. Stone
James Stone

John Elliott

Date: Dec. 13, 1997

Abstract

Phosphatidylcholine (PC) constitutes the predominant phospholipid in eukaryotic cell membranes. In mammals, every tissue synthesizes PC via the CDP-choline pathway, using choline as the initial substrate. The liver contains a second pathway for the synthesis of PC, the methylation of phosphatidylethanolamine (PE). This pathway, believed to account for between 20 and 40 % of hepatic PC, is catalyzed by the enzyme phosphatidylethanolamine *N*-methyltransferase (PEMT).

This thesis represents an attempt to answer this question: "Since the liver contains the ability to synthesize PC via the CDP-choline pathway, why has this organ maintained a second pathway, the methylation of PE?" The first step involved the cloning and characterization of the mouse gene encoding PEMT2, the mitochondria-associated membrane isoform of PEMT. The gene, originally named *Pempt2*, was found to consist of seven exons and six introns spread over at least 35 kb. The gene was mapped to mouse chromosome 11, approximately 31 centimorgans from the centromere.

Knowledge of the structure of the gene allowed the construction of a vector for the targeted disruption of *Pempt2*. Animals carrying two copies of the disrupted gene completely lacked PEMT2 protein. As well, PEMT activity *in vitro* and *in vivo* was almost completely eliminated, indicating that all PEMTs were encoded by this gene, hence its renaming to *Pempt*. Very little effect on hepatic phospholipid composition from the elimination of PE methylation was observed. However, the activity of the membrane-bound form of CTP:phosphocholine cytidylyltransferase, the rate-limiting enzyme of the CDP-choline pathway, was

60% higher in *Pempt* (-/-) mice compared to their normal littermates, suggesting that the CDP-choline pathway is stimulated to compensate for the lack of PEMT. The mice lacking PEMT displayed no obvious pathophysiological defects, nor any differences in lipoprotein profile or bile composition compared to normal animals.

The effects of feeding the mice a choline-deficient diet were examined. In *Pempt* (-/-) mice, this resulted in massive fatty liver, altered lipoprotein profile, bile accumulation in the gall bladder, and severe (probably lethal) liver damage. PC levels were reduced in the liver, plasma and bile. This result demonstrates the consequences of eliminating net PC biosynthesis and the importance of PEMT as an endogenous source of choline.

Finally, the cloning and characterization of human liver PEMT2 cDNAs is described. At the protein level, human and rat PEMT2 (previously cloned) were 80% identical and 90% similar. Alternative-splicing of the 5'-untranslated region was suggested, perhaps in a tissue-specific manner. The human PEMT2 gene mappped to chromosome 17p11.2. These experiments represent an initial step in examining the role of PEMT in human hepatocellular carcinoma.

Table of Contents

CHAPTER 1 - Introduction to Phosphatidylcholine Biosynthesis 1

1.1 Phosphatidylcholine Structure and Function 2

1.2 Biosynthesis of PC: the CDP-choline pathway 7

1.2.1 Choline kinase 8

1.2.2 CTP:phosphocholine cytidylyltransferase 9

1.2.3 Cholinephosphotransferase 12

1.3 Biosynthesis of PC: the PE methylation pathway 14

1.3.1 Mammalian PEMT: enzyme purification and cDNA cloning 15

1.3.2 PEMT across the kingdoms 17

1.3.2(a) Bacteria 17

1.3.2(b) Yeast and other eukaryotic microbes 18

1.3.2(c) Plants 22

1.3.3 Regulation of PEMT activity 23

1.3.3(a) Substrate regulation 23

1.3.3(b) Hormonal regulation 24

1.3.3(c) Regulation during development 25

1.3.3(d) Coordinate regulation of PE methylation and the CDP-choline pathway 26

1.3.4 Physiological functions of PE methylation 27

1.3.4(a) PE methylation as a source of choline 28

1.3.4(b) PEMT-derived PC is targeted for secretion? 28

1.3.4(c) PE methylation as a source of specific molecular species of PC 30

1.3.4(d) Is PEMT as tumour suppressor? 31

1.3.5 PEMT controversies 32

1.3.5(a) How many enzymes? 33

1.3.5(b) Is PEMT expressed in extrahepatic tissues? 34

1.3.5(c) Does PE methylation play a role in intracellular signaling? 35

1.4 Choline 37

1.4.1 Roles of choline 37

1.4.1(a) Choline as a precursor of PC 37

1.4.1(b) Choline incorporation into acetylcholine 37

1.4.1(c) Choline contribution to the 1-carbon pool 38

1.4.2 Choline deficiency 38

1.5 Thesis objective 42

References 51

CHAPTER 2 - Characterization of the murine phosphatidylethanolamine *N*-methyltransferase-2 gene 73

2.1 Introduction 74

2.2 Materials and Methods 75

2.2.1 Isolation and characterization of genomic clones 75

2.2.2 Southern blotting 76

2.2.3 RNA primer extension assay 76

2.2.4 Reverse transcriptase-PCR 77

2.2.5 PEMT2 mRNA and protein analysis 77

2.2.6 Chromosome mapping 78

2.3 Results 78

2.3.1 Cloning and characterization of the mouse PEMT2 78

gene

2.3.2 Mapping of the transcription initiation sites 80

2.3.3 Gene product analysis 81

2.3.4 Chromosomal localization of PEMT2 82

2.4 Discussion 83

References 99

CHAPTER 3 - Disruption of the murine gene encoding phosphatidylethanolamine *N*-methyltransferase 101

3.1 Introduction 102

3.2 Materials and Methods 104

3.2.1 Generation of phosphatidylethanolamine *N*-methyltransferase-deficient mice 104

3.2.2 Tissue collection 105

3.2.3 Determination of enzyme mass and activity 105

3.2.4 Phospholipid analyses 106

3.2.5 *In vivo* metabolism of [^{3}H]methionine 107

3.3 Results 107

3.3.1 Generation of *Pempt2*-deficient mice 107

3.3.2 Phosphatidylethanolamine *N*-methyltransferase-2 is absent in *Pempt2* (-/-) mice 108

3.3.3 PC and PE levels in the liver are minimally affected by elimination of *Pempt2* 109

3.3.4 CTP:phosphocholine cytidylyltransferase activity is increased in the livers of *Pempt2*-disrupted mice 109

3.3.5 Incorporation of [3H]methionine into choline-containing phospholipids 110

3.3.6 Mice homozygous for the disruption of *Pempt2* display no obvious physiological defects 111

3.4 Discussion 112

References 123

CHAPTER 4 - Choline-deficiency causes massive liver damage in mice lacking PE methylation 126

4.1 Results and Discussion 127

References 166

CHAPTER 5 - cDNA cloning and chromosomal localization of human PEMT2 168

5.1 Introduction 169

5.2 Materials and Methods 171

5.2.1 Screening a human liver cDNA library 171

5.2.2 Chromosomal mapping of the human PEMT2 gene 171

5.3 Results 172

5.3.1 Cloning of a human cDNA for PEMT2 172

5.3.2 Chromosomal localization of the human PEMT2 gene 174

5.4 Discussion 175

References 186

CHAPTER 6 - Summary, Conclusions and Future Studies 188

6.1 Summary and Conclusions 189

6.2 Future studies 192

6.2.1 Remnant PE methylation activity 192

6.2.2 PEMT at weaning 193

6.2.3 The role of PEMT in lipoprotein secretion 193

6.2.4 The role of PEMT in generating unsaturated PC 194

6.2.5 The influence of PEMT on choline metabolism 195

6.2.6 PEMT during fasting 196

6.2.7 The progression and reversibility of liver damage in *Pempt* (-/-) mice fed a choline-deficient diet 196

6.2.8 The consequences of choline-deficiency in non-hepatic tissues of *Pempt* (-/-) mice 197

6.2.9 The role of PEMT as a tumour suppressor 197

References 198

APPENDIX 1 - Does the liver-enriched nuclear factor C/EBPα stimulate *Pempt* transcription 200

App. 1.1 Introduction 201

App. 1.2 Materials and Methods 204

App. 1.2.1 Isolation of primary rat hepatocytes 204

App. 1.2.2 Preparation and analysis of RNA 204

App. 1.2.3 Sequencing of the 5' upstream region of the *Pempt* gene 205

App. 1.2.4 Construction of reporter vectors 205

App. 1.2.5 Maintenance and transfection of cultured cells 206

App. 1.2.6 Enzyme and protein detection and measurement 206

App. 1.3 Results 207

App. 1.3.1 *Pempt* gene expression in primary hepatocytes 207

App. 1.3.2 Co-transfection of C/EBPα and *Pempt* promoter reporter constructs 208

App. 1.3.3 Immunoblot of proteins from C/EBPα-transfected cells 211

App. 1.4 Discussion 211

References 222

List of Tables

Table 2.1 Exon/intron organization of the mouse PEMT2 gene 98

Table 4.1 Choline-deficiency in *Pempt* (-/-) mice causes aminotransferase leakage into the bloodstream 165

List of Figures

Figure 1.1 Structure of phosphatidylcholine 44

Figure 1.2 The CDP-choline pathway for the biosynthesis of PC 46

Figure 1.3 The PE methylation pathway for the biosynthesis of PC 48

Figure 1.4 The metabolic fates of choline 50

Figure 2.1 The mouse PEMT2 gene 87

Figure 2.2 Putative mouse PEMT2 cDNA based on exon sequences 89

Figure 2.3 Southern blotting of clone 2614 and mouse genomic DNA probed with rat PEMT2 cDNA 90

Figure 2.4 Primer extension and reverse transcriptase-PCR results indicate two close transcription start sites 92

Figure 2.5 The PEMT2 gene product 93

Figure 2.6 *Pempt2* maps to mouse chromosome 11 95

Figure 2.7 Sequence analysis of the murine PEMT2 exon 1 and its 5' flanking region 97

Figure 3.1 *Pempt2* targeting strategy 117

Figure 3.2 Elimination of methylation activity of PE, PMME and PDME in *Pempt2* -disrupted mice 118

Figure 3.3 Phospholipid composition of livers from mice of all three genotypes 119

Figure 3.4 Membrane-bound CT activity was increased in *Pempt2* knockout mice 120

Figure 3.5 Incorporation of radiolabeled methionine into choline-containing phospholipids 122

Figure 4.1 Mice lacking PEMT fed a choline-deficient diet lost weight 140

Figure 4.2 Grossly enlarged liver in *Pempt* (-/-) mice fed a choline-deficient diet 142

Figure 4.3 Extensive vacuolization in liver sections from *Pempt* (-/-) mice fed a choline-deficient diet 144

Figure 4.4 Oil Red O staining of liver sections 145

Figure 4.5 Hepatic phospholipids, and particularly PC, were reduced in *Pempt* (-/-) mice fed a choline-deficient diet 147

Figure 4.6 CT activity in choline-supplemented and choline-deficient mice 149

Figure 4.7 Liver triacylglycerol 150

Figure 4.8 Free cholesterol accumulates in the livers of *Pempt* (-/-) mice fed a choline-deficient diet 151

Figure 4.9 Plasma triacylglycerol levels (measured as glycerol) in mice fed choline-deficient and choline-supplemented diets 152

Figure 4.10 Plasma total cholesterol levels in mice fed choline-deficient and choline-supplemented 153

Figure 4.11 Plasma phospholipid levels in mice fed choline-deficient and choline-supplemented diets 154

Figure 4.12 Plasma lipoprotein profiles from mice fed a choline-deficient diet 156

Figure 4.13 Bile accumulates in the gall bladder of *Pempt* (-/-) mice fed a choline-deficient diet 157

Figure 4.14 Bile acid concentration in the bile of mice fed choline-deficient and choline-supplemented 158

Figure 4.15 Cholesterol concentration in the bile of mice fed choline-deficient and choline-supplemented diets 159

Figure 4.16 PC concentration in the bile of *Pempt* (-/-) mice fed a choline-deficient diet was markedly reduced 161

Figure 4.17 AdoMet and AdoHcy in the livers of mice fed choline-deficient and choline-supplemented diets 163

Figure 4.18 The level of bile acids in the plasma is elevated in *Pempt* (-/-) mice fed a choline-deficient diet 164

Figure 5.1 Nucleotide sequence of human liver PEMT2 cDNA 180

Figure 5.2 Variation in the 5'-untranslated region of the cDNA clones 181

Figure 5.3 Comparison of human and rat PEMT2 protein sequences 182

Figure 5.4 Hydrophobicity analysis of human PEMT2 183

Figure 5.5 The human PEMT2 gene maps to chromosome 17p11.2 184

Figure 5.6 Possible structure of the human PEMT2 gene 185

Figure App. 1.1 Sequence of the proximal promoter region of the mouse *Pempt* gene 216

Figure App. 1.2 *Pempt* gene expression is reduced in isolated hepatocytes 217

Figure App. 1.3 C/EBPα stimulates transcription from the *Pempt* promoter 219

Figure App. 1.4 Mutation of -37 to +127 region of the *Pempt* gene does not affect C/EBPα-dependent induction of transcription 220

Figure App. 1.5 McArdle 7777 cells transfected with a C/EBPα expression plasmid do not express the native form of this protein 221

Abbreviations

AdoMet	*S*-adenosyl-L-methionine
ATP	adenosine triphosphate
BAC	bacterial artificial chromosome
cAMP	cyclic adenosine monophosphate
C/EBP	CCAAT/Enhancer binding protein
CD	choline-deficient
cDNA	complementary DNA
CDP-choline	cytidine diphosphocholine
ChAT	choline acetyltransferase
CK	choline kinase
CMP	cytidine monophosphate
CPT	CDP-choline: 1,2-diacylglycerol cholinephophotransferase
CS	choline-supplemented
CT	CTP:phosphocholine cytidylyltransferase
CTP	cytidine triphosphate
DAG	diacyglycerol
dbEST	Database of Expressed Sequence Tags
DME	Dubelcco's Modified Eagle's medium
DMEth	dimethylethanolamine
DNA	deoxyribonucleic acid
DOTAP	*N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethyl-ammonium methylsulfate
ER	endoplasmic reticulum
ES	embryonic stem

EST	Expressed Sequence Tag
FBS	fetal bovine serum
HDL	high-density lipoprotein
HNF	Hepatic Nuclear Factor
hPEMT2	human phosphatidylethanolamine *N*-methyltransferase 2
kDa	kilodalton
LDL	low-density lipoprotein
lysoPC	lysophosphatidylcholine
MMEth	monomethylethanolamine
mRNA	messenger RNA
PC	phosphatidylcholine
PDME	phosphatidyldimethylethanolamine
PE	phosphatidylethanolamine
PEMT	phosphatidylethanolamine *N*-methyltransferase
PMME	phosphatidylmonomethylethanolamine
PS	phosphatidylserine
RNA	ribonucleic acid
rPEMT2	rat phosphatidylethanolamine *N*-methyltransferase 2
SDS	sodium dodecyl sulfate
SMS	Smith-Magenis syndrome
STS	sequence tagged site
VLDL	very low-density lipoprotein
YAC	yeast artificial chromosome

Chapter 1

Introduction to Phosphatidylcholine Biosynthesis

1.1 Phosphatidylcholine Structure and Function

Phosphatidylcholine (PC) is the most abundant phospholipid in eukaryotic cells, comprising between 25 and 55% of the total lipid composition of cell membrane depending on the species and the tissue (1). PC was first described by Gobley in 1847 as a component of egg yolk, and named 'lecithin' (2). Soon after, Diaconow and Strecker described the basic structure of lecithin (Fig. 1.1): two fatty acyl moieties attached to a glycerol backbone and a phosphodiester linkage to choline through the third hydroxyl group (3,4). More than eighty years passed until Baer and Kates synthesized PC chemically in 1950 (5), confirming its structure and spurring the biochemical study of this important molecule.

Like all phospholipids, PC has an amphipathic nature. The hydrophobicity of the PC molecule is conferred by the two *n*-acyl chains attached by ester linkage to the glycerol backbone. The hydrophilicity is conferred by the polar phosphocholine moiety also attached to the glycerol backbone. The positive charge of the choline moiety and the negative charge of the phosphodiester result in an overall charge of 0 for PC. In an aqueous solution, PC molecules will spontaneously form a bilayer (6).

PC is found in nature with a variety of fatty acids attached to the glycerol backbone. Typically, saturated fatty acids are found at the *sn-1* position and unsaturated fatty acids are found at the *sn-2* position (7-9). In the rat liver, the most common fatty acids at the *sn-1* position are palmitate (16:0) and stearate (18:0) (10). At the *sn-2* position, the most common fatty acids are oleate (18:1Δ9), linoleate (18:2Δ9,12) and arachidonate (20:4Δ5,8,11,14). Compared to other phospholipids, notably phosphatidylethanolamine (PE) and

phosphatidylserine (PS), PC contains lower levels of hexaenoic species. In some cells of myeloid origin, between 30 and 70% of the PC has an ether linkage (O-alkyl) at the *sn-1* position (11,12). As well, heart and other electrically active tissues contain high levels (~30%) of PC as plasmalogens, with a vinyl linkage (alkyl-1-enyl) at the *sn-1* position (13).

As the predominant phospholipid, PC plays a crucial role in the maintenance of the lipid bilayer that forms the basis of cell membranes. These membranes form not only the outer edge of the cell (the plasma membrane), but also define organelles, intracellular structures that carry out specialized functions. Interestingly, PC content varies among different subcellular fractions. For example, in the rat liver, the plasma membrane contains 35% PC, while the nuclear membrane contains 60% PC (14). The low PC content, and high cholesterol content, of the plasma membrane may serve to afford this particular bilayer a higher resistance to fluidity changes caused by external factors such as changing temperatures (15). The permeability of lipid bilayers allows the rapid transport of water and small molecules across cell membranes. As well the lipid bilayers are studded with a wide variety of proteins, leading to the description of cell membranes as "fluid mosaics" (16). These proteins include receptors for extracellular signals, transporters for large molecules and biosynthetic enzymes for the components of the lipid bilayer itself.

Phosphatidylcholine plays more than just a structural role within cells. The liver and intestine secrete PC into plasma as a component of lipoproteins. PC and lysoPC form a monolayer surrounding the neutral lipid (triglycerides and cholesterol esters) core of lipoproteins, helping to solubilize it (17). As well, PC is a substrate for the enzyme lecithin:cholesterol acyltransferase, which

transfers fatty acids from the *sn-2* position of PC in lipoproteins to cholesterol to form cholesteryl esters. In rats, 64% of lipoprotein phospholipid is PC, and 23% is lysoPC (18). As well, the liver secretes PC into bile, where it forms micelles with bile salts (19). PC in bile forms micelles with bile acids, protecting the bile duct epithelium from the detergent action of bile acids (20), and facilitating the secretion of cholesterol (21) and organic anions (22). In the intestine, the phospholipid/bile acid micelles aid the absorption of dietary fat, while providing the intestine with essential fatty acids (23). Biliary phospholipids may also be required for chylomicron assembly in lymph (24). In most species, over 95% of the phospholipid in the bile is PC, with the remainder being composed primarily of PE and sphingomyelin (25). The majority of this PC consists of palmitate (16:0) and linoleate (18:2) at the *sn-1* and *sn-2* positions respectively. The importance of PC in bile is demonstrated by mice in which the *mdr2* gene, which encodes a putative PC translocase on the canicular surface of hepatocytes, has been disrupted (21,26). These mice completely lack PC in their bile and suffer from acute liver disease. Finally, PC, in particular the saturated species dipalmitoyl PC, is a critical component of lung surfactant, which serves to stabilize the lung by reducing the surface tension at the air liquid interface of the alveolar surface following the formation of a phospholipid monolayer (27).

More recently, the function of PC as a source of intracellular signals has become known. The stimulation of phospholipase A_2 by circulating hormones, growth factors and other agonists, including bradykinin and thrombin, releases arachidonate, the precursor to bioactive eiconasoids, from the *sn-2* position of PC (28). The other product of this deacylation reaction, lysoPC, may also have a signaling function in cells (29). A wide variety of agonists can also induce the

hydrolysis of PC by phospholipases C and D. The primary function of these pathways appears to be the long-term generation of diacylglycerol (DAG), which serves to activate protein kinase C, which in turn through the phosphorylation of other proteins, can trigger a wide variety of intracellular responses. Several recent reviews cover this fascinating and complex aspect of PC metabolism (30-35).

Phosphatidylcholine also serves as a substrate for enzymatic reactions that generate other bioactive lipids. PC reacts with ceramides to generate another choline-containing lipid, sphingomyelin, releasing DAG in the process. The hydrolysis of sphingomyelin can be stimulated by extracellular factors to release ceramides, which are believed to function as apoptotic signals, initiating programmed cell death, and as mitogens, stimulating cell division (36,37). Which stimulatory pathway ceramides activate may depend on the cell type, and the co-activation of other signaling pathways. PC with an ether-linked alkyl group at the *sn-1* position is a precursor of platelet-activating factor (PAF; 1-alkyl-2-acetyl-sn-glycero-3-phosphocholine) (38). A variety of cell types generate PAF in response to agonists (39). PAF acts by stimulating the aggregation of platelets and producing an anti-hypertensive effect. Other lipids for which PC can serve as a precursor, including phosphatidic acid and lysophosphatidic acid (40-42) and sphingomyelin derivatives (43-45), have also been shown to have potent mitogenic effects.

Clearly, phosphatidylcholine is a crucial molecule, both as a structural component of cell membranes, lipoproteins, bile and lung surfactant, and as a source of intracellular messengers. There are no known genetic diseases in humans or other mammals in which PC biosynthesis is impaired, presumably

because such an impairment would be lethal very early on in embryonic development. In Chinese hamster ovary cells in which PC biosynthesis is obliterated by a temperature-sensitive mutation, incubation at the restrictive temperature is lethal (46). Interestingly, this death occurs by apoptosis (47). Therefore, perhaps the most elegant evidence demonstrating the importance of PC is the fact that mammalian organisms cannot tolerate its absence.

1.2 Biosynthesis of PC: the CDP-choline pathway

As the predominant phospholipid in eukaryotes, the biosynthesis of PC has been studied extensively. There are four pathways for the biosynthesis of PC: the CDP-choline pathway, the PE methylation pathway, the reacylation of lysoPC and base exchange for choline. Only the first two pathways will be discussed here.

The CDP-choline pathway was first described in the 1950´s, following the work of Eugene Kennedy and co-workers (48). For this reason, the pathway is occasionally referred to as the Kennedy pathway. It consists of three steps: 1) the phosphorylation of intracellular choline by choline kinase (CK) to form phosphocholine, 2) the reaction of phosphocholine with cytidine triphosphate (CTP) to form cytidine diphosphocholine (CDP-choline), catalyzed by the enzyme CTP:phosphocholine cytidylyltransferase (CT), and 3) the exchange of cytidine monophosphate for DAG catalyzed by CDP-choline:1,2-diacylglycerol cholinephosphotransferase (CPT), which produces PC (Fig. 1.2). This pathway occurs in all nucleated cells, and is the main source of PC in mammals. In a dividing cell, the biosynthesis of PC by this pathway is regulated to ensure an adequate supply for the membranes of the daughter cells (49-51). Recently, Tercé *et al.* have presented evidence suggesting that the biosynthesis of PC itself regulates the cell cycle (52). Despite the different subcellular localizations of these enzymes (discussed below), the intermediate metabolites of the pathway (phosphocholine and CDP-choline) are not freely diffusible, but instead appear to be channeled (53,54). Exogenous phosphocholine and CDP-choline are not incorporated into PC. The rate of PC catabolism is closely linked

to the rate of PC biosynthesis through this pathway, in order to maintain PC homeostasis (55-57).

1.2.1 Choline Kinase

The first step in the biosynthesis of PC is catalyzed by choline kinase (CK) (EC 2.7.1.32), which phosphorylates choline to form phosphocholine. This activity was first detected in yeast extracts by Wittenberg and Kornberg in 1953 (58). The enzyme has been purified to homogeneity from rat kidney (59), liver (60) and brain (61), with apparent subunit molecular masses of 42 kDa, 47 kDa and 47 kDa respectively. Gel filtration experiments suggest that the kidney and brain forms are dimers and the liver form a tetramer. In all cases, CK is cytosolic, and requires ATP and Mg^{2+} ions for activity. Immunoblots of kidney tissue detect more than one protein with antibody against the rat liver choline kinase; this result and other evidence suggests that multiple isoforms of choline kinase exist, some of which are immunologically distinct (60,62,63). Interestingly, in both yeast and mammals, CK also phosphorylates ethanolamine, the first step in the CDP-ethanolamine pathway for the biosynthesis of PE (60,61,64).

cDNAs encoding choline kinase have been cloned from yeast (65), rat liver (64) and human glioblastoma cells (66). The deduced molecular mass of the yeast gene product (called *CKI*) is 66 kDA. The deduced molecular mass of the rat isoform, called CK-R1, is 49.7 kDA, reasonably close to the experimental value of 47 kDa. A second rat liver isoform, called CK-R2, with a slightly smaller molecular mass has also been cloned (67). The isoform purified from rat brain, called CK-P, remains uncloned. The cloned human CK isoform has a deduced molecular mass of 52 kDa. As well, the 5′ end of the rat CK-R gene has been

cloned (67). Analysis of this genomic DNA clone reveals extensive alternative splicing, which may be a source of the multiple isoforms observed.

Although it catalyzes the first committed step in the biosynthesis of PC, choline kinase is not generally considered rate-limiting in the pathway. Nonetheless, increased CK activity can be induced by the addition of hormones (68,69), mitogens (70,71), carcinogens(63), hepatotoxins (63), and by *ras*-transformation (72-74), and in many cases this increase can be correlated to an increase in PC biosynthesis. Therefore, expression of CK appears to be regulated. Some evidence suggests that this regulation may occur in an isoform-specific manner (63,67).

1.2.2 CTP:phosphocholine cytidylyltransferase

The second step in this pathway, the conversion of phosphocholine to CDP-choline, is catalyzed by the enzyme CTP:phosphocholine cytidylyltransferase (CT) (EC 2.7.7.15). As the name suggests, CTP is the source of the cytidine base. Even though CT does not catalyze either the first committed step of this pathway, or a metabolic branch point, this step is widely believed the rat-limiting step in biosynthesis of PC in liver, lung and a variety of cell lines (75-77).

CT exists in two forms in the cell: soluble and membrane-bound. In enzyme assays, the soluble fraction requires the addition of exogenous lipids for activity; the membrane-bound fraction does not. Experiments with a wide variety of agonists and treatments suggest that increases in PC biosynthesis are correlated with an increase in the activity of CT in membrane fractions, but not

cytosolic fractions (78). This observation led to the translocation hypothesis: Agents that increase the rate of PC biosynthesis stimulate the transfer of CT from an inactive, soluble form to an active, membrane-bound form (78,79).

The mechanism of this translocation has been the subject of intense study, summarized in recent reviews (80,81). Briefly, phosphorylated CT is typically found in the soluble fraction, and dephosphorylated CT is found in the membrane fraction. However, *in vitro* studies suggest that dephosphorylation is not a prerequisite for translocation (82). As well, the addition of exogenous fatty acids and the generation of DAG stimulate translocation to the membrane (83,84). Finally, a deficiency of PC in the membrane also stimulates translocation to the membrane, suggesting a feedback loop for the regulation of PC biosynthesis (85). Therefore, it has been proposed that CT translocation is governed by the ratio of bilayer-forming lipids (e.g. PC) to non-bilayer-forming lipids (e.g. DAG) (86,87).Since the translocation hypothesis was first proposed, several other enzymes have been found to be subject to this regulatory mechanism, including phosphatidate phosphohydrolase, diacylglycerol kinase and protein kinase C.

After numerous attempts, CT was finally purified to homogeneity from rat liver in the late 1980's (88,89). Under denaturing conditions, purified CT appears as single subunits of 42 kDA (90,91). Under non-denaturing conditions, purified CT appears as a homodimer when it is bound to lipid vesicles (91). Once purification was achieved, progress was rapid. In 1990, a cDNA encoding CT was cloned from rat liver, based on the partial amino acid sequence of the purified protein (92). The calculated molecular mass based on the cDNA sequence is 41.7 kDa. Since then, cDNAs for CT have been cloned

from rat fetal lung type II cells (93), mouse testis (94), CHO cells (95), and a human cell line (96). The yeast CT gene was cloned by complementation of the *CCT* gene mutant (97,98). The murine gene for CT, named *Ctpct*, has been cloned and mapped to mouse chromosome 16 (94,99).

When the dual nature of CT was first described, "soluble" was understood to be "cytosolic", and "membrane-bound" was understood to be "microsomal". Microsomes for CT assays are generally prepared by homogenization of the tissue or cell followed by a low-speed centrifugation (100 -10000 x g) to remove nuclei and unbroken cells, and a high-speed centrifugation (100000 - 150000 x g) to separate the cytosol from membranes (100,101). Hence, the microsomes consist of a mixture of membranes from the endoplasmic reticulum, Golgi apparatus, plasma membrane and other organelle membranes. More precise subcellular localization studies with rat liver and skeletal muscle homogenates and Krebs-II ascite cells place the majority of CT activity on the ER and Golgi apparatus, and the sarcoplasmic reticulum in muscle, with minimal activity on the plasma membrane and other organelles (102,103). In contrast, in several cell lines, CT is found in the nucleus, using immunofluorescence techniques and Percoll gradient separation (104-107). A nuclear localization signal has been identified at the N-terminus of the CT protein (108). The nuclear localization of CT may be a consequence of the rapid growth of these cells, especially in view of the link between PC biosynthesis and the cell cycle discussed above. In support of this hypothesis, in primary hepatocytes which do not undergo rapid cell division, soluble CT is primarily cytosolic (109). The sub-cellular localization of yeast CT is not known, although it appears to associate with membranes (110).

Homology between rat liver and yeast CT, as well as homology to bacterial CTP:glycerol-3-phosphate cytidylyltransferase, suggests that the catalytic core is contained in a central region of 162 (72-234) residues (92). The domain of the rat liver CT molecule responsible for membrane binding is localized at the C-terminus of the catalytic core, between residues 236 and 287 (111,112), in particular the region between residues 267 and 277 (113). The stretch between 239 and 287, which has also been implicated as the site of lipid regulation of CT (114,115), has been hypothesized to form an amphipathic α-helix (92).

The cloning of cDNAs for CT has allowed the study of its gene expression. The lack of a TATA-box at the 5′ end of the mouse CT gene suggests a "housekeeping" promoter, with a constitutive expression impervious to external stimuli (99). However, in some instances modulation of CT mRNA levels has been observed (86,93,116,117). In at least one case (118), this modulation occurs at the level of mRNA stability.

1.2.3 Cholinephosphotransferase

The final step in the CDP-choline pathway for the biosynthesis of PC is the reaction of CDP-choline with diacylglycerol to generate PC and cytidine monophosphate (CMP), catalyzed by CDP-choline:1,2-diacylglycerol cholinephosphotransferase (CPT) (EC 2.7.8.2). This step represents a branch point in the metabolism of DAG; alternative routes for DAG include incorporation into other phospholipids, and acylation to form triacylglycerol for storage and energy metabolism. It has been hypothesized that since this step introduces fatty acids into the pathway, CPT may be responsible for the particular

molecular species patterns characteristic of PC. However, in lung, CPT does not discriminate against any particular DAG species, including dipalmitin, although dipalmitoyl PC is the primary component of lung surfactant (119-123). In rat liver, CPT displays a preference for saturated fatty acids ≤17 carbons long at the *sn-1* position, but retains activity against a wide variety DAG substrates (124). Therefore, the molecular species composition of PC in a particular tissue appears to be determined partly through this reaction and partly through deacylation-reacylation. This final step in PC biosynthesis has never been found to be rate-limiting, except when CT is overexpressed (57).

Initial subcellular fractionation studies placed CPT on the ER (125). However, more recent evidence suggests that enzyme may also be found on the Golgi apparatus (102,126,127). The presence of CPT on the nuclear membrane alongside CT remains to be determined, although given the channeling of PC metabolites (54), this seems to be a reasonable hypothesis.

CPT has never been purified, most likely because it is an integral membrane protein, and extremely difficult to extract with detergents without destroying its activity (103). However, the yeast gene, *CPT1*, has been cloned by complementation of a mutant deficient in this activity(128). Recently, this cDNA has been used as a probe for screening Expressed Sequence Tag (EST) databases of mammalian cDNAs *in silico*, with some success (C. McMaster, personal communication).

1.3 Biosynthesis of PC: the PE methylation pathway

In the early 1940s, separate groups using radiolabeled precursors determined that both methionine and ethanolamine are biosynthetic precursors for choline in rats(129,130). Work by Bremer and Greenberg in the late 1950s and early 1960s shows that PE rather than an aqueous metabolite of ethanolamine is the methyl group acceptor(131,132), that *S*-adenosylmethionine (AdoMet) is the methyl group donor(133), and that the reaction proceeds via phosphatidylmonomethylethanolamine (PMME) and phosphatidyldimethylethanolamine (PDME) intermediates (134). Thus, the second pathway for the biosynthesis of PC, three sequential *N*-methylations of PE, is outlined (Figure 1.3). The enzyme that catalyzes the three transmethylation reactions is phosphatidylethanolamine *N*-methyltransferase (PEMT) (EC 2.1.1.17). These reactions occur in a concerted fashion; there is no accumulation of the intermediates PMME and PDME (135). Hence the first methylation is the rate-limiting step(131,136).

Soon after the their elucidation of the PEMT pathway in rats, Bremer and Greenberg discovered that the liver is the only organ capable of carrying out PE methylation to a meaningful extent (133). Other tissues contain less than 6% the *in vivo* PEMT activity that liver does. Subcellular fractionation studies place PEMT, like other phospholipid biosynthesis enzymes, on the cytosolic surface of microsomal membranes (137-139). By comparing the rate of radiolabeled methionine incorporation into PC to the rate of radiolabeled glycerol incorporation into PC in isolated rat hepatocytes, Sundler and Åkesson estimated that the rate of formation of PC via PE methylation is 20 to 40% of the total rate of PC formation, depending on the addition of exogenous choline and

fatty acids (140). These calculations are by no means definitive, since they are based on *in vitro* culture conditions that may not accurately reflect *in vivo* conditions, nor do they account for the possibility that PC derived from the different pathways may be turned over or secreted at different rates. By following the metabolic fate of radiolabeled choline and ethanolamine injected into rats, others have estimated that the PE methylation pathway forms PC at 3.5 (141) to approximately 10% (142) the rate that the CDP-choline pathway does. However, these studies do not account for the turnover of PEMT-derived PC and re-incorporation of the labeled choline into PC via the CDP-choline pathway, nor do they account for PS decarboxylation as a possible preferred source of PE for methylation. Interestingly, in rat hepatocytes, newly-made PE from the CDP-ethanolamine pathway appears to be the preferred substrate for methylation (143,144). In contrast, in the newborn guinea pig liver, there does not appear to be a preference for newly-synthesized PE over bulk PE as a substrate for methylation (145). PE generated by the decarboxylation of PS is also a substrate for methylation in primary rat hepatocytes (146). In the rainbow trout, the contribution of PE methylation to total PC biosynthesis is greater at 5°C than at 20°C (147). Today, it has become commonly accepted that 20-40% of PC synthesis in the liver can be accounted for by PE methylation (148) .

1.3.1 Mammalian PEMT: enzyme purification and cDNA cloning

PEMT was first purified to homogeneity by Ridgway and Vance in 1987 (149). The purification took advantage of the ability of Triton X-100 to solubilize PEMT without inhibiting its activity (136). The final product was an 18.3 kDa protein with final specific activities of 0.63, 8.59 and 3.75 μmol/min/mg protein for PE, PMME and PDME substrates respectively, all at the same pH optimum of

10. Kinetic studies suggest one phospholipid binding site (150). The purified enzyme displays a higher rate of methylation with synthetic PEs containing unsaturated fatty acids, particularly with two or more double bonds, while disaturated PEs are not methylated at all (151). The inclusion of other non-substrate phospholipids, particularly polyunsaturated PCs, in the Triton micelles of the PEMT assay increases activity towards disaturated and monounsaturated PEs (150,151), suggesting that specific lipid phase properties are required to generate maximum activity with a PE substrate.

Using oligonucleotides based on the N-terminal amino acid sequence of the purified PEMT (152), a cDNA with one open reading frame encoding a 199-amino acid protein with a calculated molecular mass of 22.3 kDa was cloned from a rat liver library (153). The predicted protein sequence contains four hydrophobic stretches that may correspond to transmembrane segments. As well, the predicted protein sequence from the 5′ end of the open reading frame matches that of the purified protein. This sequence is 44% identical and 68% similar to the yeast *PEM2* protein (154). Expression of the cDNA in McArdle RH7777 rat hepatoma cells, COS-1 cells and baculovirus-infected Sf9 cells results in several-fold increases in PEMT activity, using PE, PMME and PDME as substrates. The kinetic parameters and pH optimum of the expressed enzyme closely match those of the purified PEMT. An antibody to a peptide based on the presumed C-terminus of the cDNA-encoded protein detects a major band corresponding to a molecular mass of approximately 20 kDa in an immunoblot of liver total homogenates, and in homogenates of cells expressing the cDNA. No band is present in total homogenates of other tissues. This evidence demonstrates that the cDNA clone encodes PEMT.

When subcellular fractions of liver rat liver homogenates are prepared and assayed, an intriguing dichotomy is revealed: PEMT activity resides primarily on the ER, but the antibody based on the C-terminus of the cDNA clone detects PEMT only in the mitochondria associated membrane (MAM). This subcellular fraction, isolated from crude mitochondria by centrifugation through a Percoll gradient and believed to be a specialized subsection of the ER, is enriched in enzymes responsible for phospholipid biosynthesis (155). Immunocytochemistry confirms this subcellular localization. This result led to the identification of two PEMTs: PEMT1, localized to the ER and catalyzing the majority of PE methylation, and PEMT2, localized to the MAM. The origin and differences between the two PEMTs are not known, nor is the physiological relevance of having two. However, PEMT1 and PEMT2 can be separated on the basis of differential binding to DEAE-Sepharose (A. Noga, personal communication)

1.3.2 PEMT across the kingdoms

1.3.2(a)Bacteria

The first lesson a student of phosphatidylcholine biosynthesis learns is that PC is an exclusively eukaryotic phospholipid. However, there are a few species of bacteria that contain PC. Interestingly, they all synthesize PC by the methylation of PE. The best studied of these bacteria is *Rhodobacter* (or *Rhodopseudomonas*) *sphaeroides*, where approximately 27% of the total phospholipid is PC (156). PEMT from *R. sphaeroides* was first described as a cytosolic protein by Cain *et al.*, a surprising localization for a phospholipid biosynthesis enzyme(157). A mutant in PEMT activity, identified by its complete

lack of PC, was isolated, allowing complementation cloning of the affected gene, named *pmtA* (156). The *pmtA* gene encodes a 22.9 kDa protein, which when expressed in *Eschrichia coli* generates PC. The exact function of this gene is *R. sphaeroides* remains unclear, especially since no adverse affects are observed in the *pmtA* mutant. However it is interesting to note that *R. spaeroides*, unlike most bacteria, contains internal membrane structures. The presence of PC may be correlated with this fact.

Other bacteria that methylate PE include *Agrobacterium tumefaciens*, from which PEMT has been partially purified (158), *Clostridium beijerinckii*, where the methylation proceeds only to PMME (159), *Zymonas mobilis*, from which PEMT has been purified to homogeneity (160), and several others (161,162). The reason why these particular species synthesize PC, and others do not, remains unknown.

1.3.2(b)Yeast and other eukaryotic microbes

Yeast, in particular the baker's yeast *Saccharomyces cerevisiae*, has proven to be a valuable model organism for the study of eukaryotic metabolism, due to its well-characterized biochemistry and molecular genetics, and its ease of cultivation on chemically-defined media. In particular, the generation of mutants with easily observed phenotypes has allowed cloning by complementation, without the need for purification of the enzyme. This technique has been useful for the elucidation of PC biosynthetic pathways in yeast. Interestingly, *S. cerevisiae* has an unusually high tolerance for low levels of PC (163). This fact has complicated the study of PC biosynthesis in this

organism, by allowing survival of mutants under conditions that one might expect to be lethal.

Yeast methylate PE to generate PC (164). As well, yeast can also synthesize PC via the CDP-choline pathway when exogenous choline is present (165). In the absence of choline, the CDP-choline pathway serves primarily to recycle choline from PC turnover (166). Surprisingly, mutagenesis of *S. cerevisiae* followed by growth on choline-deficient media did not reveal mutants defective in PE methylation. However, other strains with altered PC biosynthesis were detected. The *opi3* (overproduction of inositol) strain accumulates PMME(167,168). A second mutant strain, *cho2*, was isolated as variation of the *cho1* strain, which is defective in PS synthase (169). Specifically, the *cho2* mutation imposes choline auxotrophy on the *cho1* strain which can not be overcome by the addition of exogenous ethanolamine. These yeast are impaired in their ability to methylate PE, and show reduced PC levels when cultured in the absence of choline. Tetrad analysis demonstrates that the *CHO2* and *OPI3* genes are not closely linked (169). Yamashita *et al.* have independently isolated mutant *S. cerevisiae* strains with identical phenotypes to *cho2* and *opi3* , which they name *pem1* and *pem2*.

The isolation of two strains with different phenotypes suggests that the PE methylation pathway is encoded by two genes in *S. cerevisiae*: *CHO2/PEM1* encodes an enzyme which catalyzes the methylation of PE to PMME, and *OPI3/PEM2*, encodes an enzyme that catalyzes the methylation of PMME to PC. Complementation cloning of these genes confirms this hypothesis. Two different genes were isolated, sequenced and expressed in the *pem1 pem2* double mutant (154). The cloned *PEM1* gene methylates only PE, while the cloned

PEM2 gene methylates PMME and PDME preferentially, but also methylates PE (154,170). In fact, the *PEM2* gene complements the *pem1* mutation (154,171). This redundancy may explain the lack of strict choline auxotrophy in the *cho2/pem1* strain.

PEM1 and *PEM2* encode strikingly different proteins. The *PEM1* gene product, called phosphatidylethanolamine methyltransferase by Yamashita, is a 869 amino acid protein with a calculated molecular mass of 101 kDa, a pH optimum of 9.9 and a K_m of 60 μM for AdoMet (154,170). The *PEM2* gene product, named phospholipid methyltransferase, is a 206 amino acid protein with a calculated molecular mass of 23 kDa, a pH optimum of 8.1 and a K_m of 180 to 240 μM for AdoMet, depending on the reaction step (154,170). Despite these differences, both are integral membrane proteins with stretches of significant sequence homology to each other and to other methyltransferases (154).

PE methylation has also been studied in the fission yeast *Schizosaccharomyces pombe*. These yeast share the same fundamental phospholipid biosynthetic pathways with *S. cerevisiae*, except for the absence of inositol-1-phosphate synthase (172). As well, *S. pombe* has a much lower tolerance for disruptions in phospholipid composition than *S. cerevisiae*, which greatly simplified the search for mutations in PE methylation using choline auxotrophy as a phenotype (163). Two mutant strains, *cho1*$^-$ and *cho2*$^-$, have been isolated (173), and the corresponding genes, *cho1*$^+$ and *cho2*$^+$, cloned (174). The *cho1*$^+$ gene from *S. pombe* encodes a protein that catalyzes the methylation of PMME and PDME, and the *cho2*$^+$ gene from *S. pombe* encodes a protein that catalyzes the methylation of PE only. However, unlike the

OPI3/PEM2 gene in *S. cerevisiae*, which can complement the *cho2/pem1* mutant, the *cho1*$^+$ gene in *S. pombe* can not complement the *cho2*$^-$ mutant. One explanation is that the *S. pombe cho1*$^+$ gene product has much stricter substrate requirement, PMME and PDME only, than the *S. cerevisiae OPI3/PEM2* gene product, which can methylate PE (171).

Choline auxotrophic mutants of the mold *Neurospora crassa* have also proven valuable for the study of PE methylation in this organism. Strain 47904, whose growth is inhibited in the absence of choline, accumulates PMME, and to a lesser extent, PDME (175). Strain 34486, a strict choline auxotroph, has increased levels of PE (176). Measurement of PEMT activity in these strains showed that strain 47904 is deficient in the methylation of PMME and PDME, while strain 34486 is deficient in the methylation of PE (177). Crossing these strains to generate the double mutant results in an organism unable to methylate PE at all (178). These results suggest that like the yeast discussed above, the PE methylation pathway in *N. crassa* is catalyzed by at least two enzymes. The second enzyme, the one that methylates PMME and PDME, has been purified from *N. crassa* (179).

The slime mold *Dictyostelium discoideum* methylates PE, although in this case, PMME rather than PC appears to be the major product (180,181). Kinetic data, specifically the fact that double reciprocal initial velocity plots generate two linear components with two different calculated K_m values for AdoMet, suggests that two different enzymes are involved in the pathway (180).

The phospholipid metabolism of the ciliate protozoan *Tetrahymena pyriformis* is of particular interest because of the presence of 2-

aminoethylphosphonolipid, an analogue of PE containing a phosphoester rather than a phosphodiester moiety. This lipid composes 20% of the total lipid in the organism (182). *Tetrahymena* is able to synthesize PC by both the CDP-choline pathway and PE methylation, which occurs primarily on microsomes (183). However, no methylation of 2-aminophosphonolipid occurs, suggesting that the methyltransferase is specific for a phosphoester bond joining the ethanolamine head group to the phosphate (183,184). The product of such a methylation if it occurred, *N,N,N*-trimethyl-2-aminoethylphosphonate-lipid, can be generated by incubating cultures with *N,N,N*-trimethyl-2-aminoethylphosphonate, resulting in deleterious effects on *Tetrahymena* growth rates (185).

1.3.2(c)Plants

PC biosynthesis in plants has not been studied to nearly the same extent as it has in mammals, although PC is the major phospholipid component of extrachloroplastic membranes (186). Nonetheless, some interesting facts concerning PE methylation, or lack thereof, have been uncovered, primarily by incubating plant tissues with radiolabeled methionine and ethanolamine, and determining its incorporation into aqueous and non-aqueous metabolites. In duckweed (*Lemna paucicostata*) and sugarbeet (*Beta vulgaris*), there is no methylation of PE, and little methylation of PMME or PDME (187,188). Instead, phosphoethanolamine, a precursor to PE, is methylated three times to form phosphocholine, which is then incorporated into PC via the CDP-choline pathway. However, other plants have different patterns of radiolabel incorporation. In soybean (*Glycine max*), phosphoethanolamine is methylated once, then incorporated into PMME by a Kennedy-like pathway, followed by

methylation to PDME and PC (189). Celery (*Apium graveolens*) cells in culture may also follow this pathway, but methylation of other aqueous ethanolamine derivatives has not been ruled out (190). Carrots (*Daucus carota*) and barley (*Hordeum vulgare*) use both of the above pathways (189,191). Olives (*Olea europaea*) methylate phosphoethanolamine twice, then either methylate a third time to form phosphocholine, or incorporate phosphodimethylethanolamine into PDME, which is methylated to form PC (192). *In vitro* methyltransferase activity assays on cell-free *Lemna*, carrot and soybean homogenates using the phospho bases as substrates reveal phosphoethanolamine, phosphomonomethyethanolamine and phosphodimethylethanolamine methyltransferase activity in *Lemna* and carrots, but only phosphoethanolamine methyltransferase activity in soybean (193). In all cases, AdoMet is the methyl group donor. To this date, there is no evidence that any plant is capable of catalyzing the methylation of PE (194). The reason why different plants follow different pathways for the synthesis of PC via methylation is unknown.

1.3.3 Regulation of PEMT activity

1.3.3(a) Substrate Regulation

The effects of altering the concentrations of substrates on PEMT activity *in vivo* have been studied extensively. By increasing the concentration of PE in isolated hepatocytes with the addition of ethanolamine, the rate of conversion of PE to PC is also increased (140,195). In a similar manner, the addition of monomethylethanolamine and dimethylethanolamine increases the concentration of PMME and PDME respectively, which also stimulates methylation (140).

As well, the exogenous addition of methionine to hepatocytes raises the intracellular concentration of AdoMet, thus increasing the AdoMet/AdoHcy ratio and in turn increasing the rate of PE methylation (140,196). In contrast, raising the intracellular concentration of AdoHcy by adding exogenous homocysteine lowers the AdoMet/AdoHcy ratio and inhibits PE methylation (196). Inhibitors of AdoHcy hydrolase also increase the intracellular level of AdoHcy, lower the ratio of AdoMet to AdoHcy and inhibit PE methylation (197-201). Thus modulation of the ratio of AdoMet to AdoHcy is an important control mechanism for PEMT activity *in vivo*, as it is for other methyltransferases (202).

1.3.3(b) Hormonal Regulation

The study of PE methylation in response to hormones is rife with contradictory results. Perhaps the most widely studied hormone with respect to PEMT is glucagon. This peptide hormone, which signals an energy deficit, binds to a receptor on the surface of liver cells, triggering an accumulation of the second messenger cAMP and the activation of a cAMP-dependent protein kinase. Incubation of hepatocytes with glucagon and the cAMP analogue chlorophenylthio-cAMP either increases or reduces the incorporation of radiolabeled precursors into PC via methylation, depending on the experimenter (203-205). These results are complicated by the fact that glucagon also increases the rate of PE synthesis, which may also stimulate PE methylation . In contrast, the ß-adrenergic agonist isoprenaline increases the conversion of PE to PC, in conjunction with an accumulation of cAMP (206). At the same time, *in vitro* PEMT activity from the treated cells is either unchanged or increased (204,205,207). Treatment of microsomes with ATP and cAMP

increases PEMT activity twofold (208), but treatment of microsomes with cAMP-dependent protein kinase does not affect PEMT activity (209). Still, an unidentified cytosolic kinase may increase PEMT activity (209). Purified PEMT is only weakly phosphorylated by cAMP-dependent protein kinase *in vitro*, without an effect on its enzymatic activity (210). Treatment of hepatocytes with the cAMP analogue N^6-2′-*O*-dibutryladenosine 3′, 5′ cyclic monophosphate does not result in phosphorylation of PEMT protein isolated by 2D gel electrophoresis (210).

Vasopressin and angiotensin activate PEMT twofold in hepatocyte homogenates (211). Calcium and calmodulin have the same effect (212), although calmodulin-dependent protein kinase and ATP do not activate microsomal PEMT (209). Insulin has no direct effect on PE methylation, but may antagonize glucagon effects (213). The cloning of a cDNA for PEMT and the development of an antibody against PEMT will allow the examination of these hormone effects at the level of gene expression and enzyme mass.

1.3.3(c) Regulation during development

In the developing rat liver, PEMT activity begins to increase steadily from background levels five days before birth, peaking at 10 days after birth (214,215). From this point, PEMT activity declines slightly until reaching a steady level in the adult liver. This change in activity is accompanied by an increase in the amount of PEMT2 mRNA protein detected in the liver (215). PEMT activity during the development of the rabbit liver increases with a similar pattern: activity starts to increase four days prior to birth, peaking 14 days after birth, then declining slightly (216). The appearance of PEMT activity is

correlated with the terminal differentiation of hepatocytes that occurs perinatally (217), and inversely correlated with the growth rate of the liver (215).

1.3.3(d) Coordinate regulation of PE methylation and the CDP-choline pathway

Reciprocal regulation of the PEMT and CDP-choline pathways for PC biosynthesis has been demonstrated in several cases. Long-chain fatty acids inhibit PEMT *in vivo* and *in vitro*, but stimulate translocation of CT to microsomes, elevating PC synthesis from the CDP-choline pathway (218). The inhibition of PEMT by 3-deazaadenosine also stimulates the CDP-choline pathway (197). Similar effects with methylation inhibitors are also seen in *Tetrahymena* (219). In both of these cases, CT translocation to microsomes is increased, presumably to maximize PC synthesis from choline liberated by PC turnover. Overexpression of PEMT2 by transfection of McArdle RH7777 cells also results in inhibition of the CDP-choline pathway by decreasing CT gene expression (86).

Treatments that affect the growth rate of hepatocytes also affect the relative contributions of PE methylation and the CDP-choline pathway to total PC synthesis. Partial hepatectomy, in which 70% of the liver is removed, results in proliferation of a undifferentiated subpopulation of the remaining hepatocytes to restore proper liver mass (220). In this case, PEMT2 gene expression, enzyme mass and PEMT activity are all markedly reduced, while CT gene expression, enzyme mass and activity (both total and microsomal) are all increased (221). This result is in contrast to most liver specific genes, whose expression does not change during regeneration (222). Induction of non-

neoplastic liver growth with lead nitrate also decreases PEMT activity and PEMT2 gene expression, while stimulating CT activity (223). Induction of neoplastic, i. e. tumourigenic, liver growth with diethylnitrosamine and methylnitrosourea also reduces PEMT2 enzyme mass within the tumor, while stimulating CT activity (Tessitore, Cui and Vance, unpublished observations).

In some instances, coincidental, rather than reciprocal, regulation of the PE methylation and CDP-choline pathways has been observed. Like PEMT, all the enzymes of the CDP-choline pathway are stimulated perinatally in the liver (214,224-227). CT stimulation occurs by translocation of the enzyme to microsomal membranes. The resulting burst of PC synthesis at birth is believed to be a response to the need for PC secretion into bile, in order to absorb milk lipids (214). Glucagon and cAMP analogues, which may inhibit PE methylation *in vivo*, also inhibit PC synthesis via the CDP-choline pathway (228-230). The hypolipidemic agents bezafibrate and clofibric acid inhibit PE methylation *in vivo* and PEMT *in vitro* without stimulating the CDP-choline pathway (231). In contrast, choline-deficiency (discussed below) stimulates *in vitro* PEMT activity over the short term by increasing the amount of PE available for methylation (232). Longer term choline-deficiency eventually results in the stimulation of PEMT2 gene expression, as measured by mRNA levels (233).

1.3.4 Physiological Functions of PE Methylation

One of the fundamental questions remaining to be answered in the study of PE methylation is simply "Why?" Why has the mammalian liver, which like all other tissues can synthesize PC via the CDP-choline pathway, retained PE methylation? It has been widely hypothesized that PE methylation may

generate a distinct pool of PC independent of CDP-choline-derived PC, perhaps for a specific metabolic function. Evidence that may support this hypothesis is the fact that CHO cells carrying a temperature-sensitive mutation in CT can not be rescued by expression of exogenous PEMT2, despite the restoration of PC levels (234). Exogenous expression of PEMT2 is also unable to rescue McArdle RH7777 cells deprived of choline (P. S. Vermeulen, personal communication). These results suggest that PEMT-generated PC may not be functionally equivalent to CDP-choline-derived PC. In contrast, yeast deficient in the CDP-choline pathway survive perfectly well when PE methylation is present, suggesting that the function of this pathway may differ between eukaryotic microbes and mammals (163).

1.3.4(a) PE methylation as a source of choline

One obvious possible function for PE methylation is as an endogenous source of choline, reducing the reliance on dietary choline. Choline is not only required for the synthesis of PC in extrahepatic tissues, but also the synthesis of other choline-containing molecules such as the neurotransmitter acetylcholine. The importance of choline is discussed in more detail below. To this date, PE methylation appears to be the only significant endogenous pathway for the synthesis of choline in mammals, although there are reports of phosphoethanolamine methyltransferase activity in the brain (235-237).

1.3.4(b) PEMT-derived PC is targeted for secretion

One hepatocyte-specific function that requires PC is the secretion of very low density lipoproteins (VLDL), discussed above. In fact, the biosynthesis of

PC is required for the secretion of lipoproteins (238,239). It has been hypothesized that the PC generated by PEMT is specifically targeted for lipoprotein secretion. When monolayer cultures of rat hepatocytes are incubated with radiolabeled ethanolamine, the level of radiolabeled PC in the culture medium is significantly lower than it is the cells themselves (146). However, when radiolabeled serine is added, the level of radiolabeled PC is higher in the culture medium than it is in the cells. These results suggest that methylated PE derived from PS decarboxylation, but not ethanolamine incorporation, is targeted for lipoprotein secretion. When isolated hepatocytes are treated with 3-deazaadenosine to inhibit PE methylation by 95% and radiolabeled ethanolamine added, no effect is seen on the secretion of lipoproteins or the amount of radiolabeled PC in the culture medium, although radiolabeled PE levels increase in the culture medium (240). Recently, investigators have found that under 3-deazaadenosine inhibition, as well as under PEMT inhibition by fibrates, lipidation of apoB48- but not apoB100-containing lipoproteins is reduced (241). Curiously, when isolated hepatocytes are treated with 3-deazaadenosine and radiolabeled serine added, radiolabel incorporation into cellular PC is inhibited, but radiolabel incorporation into secreted PC is unaffected (242). One explanation for this result is that there exists a second PEMT which targets PC for secretion, and is impervious to 3-deazaadenosine inhibition. The relationship between 3-deazaadenosine-sensitive and -insensitive PEMT, and PEMTs 1 and 2 remains to be determined. Hepatoma-derived cell lines that do not have endogenous PEMT activity also secrete lipoproteins (R. Lehner, personal communication). Therefore, it appears that PE methylation is not strictly required for lipoprotein secretion. However, it remains possible that PC originally derived from PS may be preferred, but not required, for secretion. Interestingly, the accumulation of

the PE methylation intermediate PMME by administration of monomethylethanolamine inhibits secretion of apoB-containing lipoproteins, although neither the PEMT nor the CDP-choline pathways is affected by this treatment (243).

The biosynthetic origin of PC secreted into bile has not been extensively studied, except with respect to fatty acid composition (244). By following the metabolism of [^{3}H]choline and [^{14}C]methionine in rats, Balint and co-workers found that both CDP-choline and PE methylation-derived PC are secreted into bile, but that methylation-derived PC forms a greater proportion of the total PC in bile than it does in hepatocytes (245). However, when PC biosynthesis is stimulated with bile acids, CDP-choline-derived PC predominates. As well, the incorporation of radiolabeled choline into PC in microsomes and its subsequent secretion into bile suggests that the CDP-choline pathway contributes at least some of the PC in bile (246). Whether the PC in bile is derived from bulk hepatocyte PC (247,248), or from a specialized pool of PC (246), remains an open question.

1.3.4(c) PE methylation as a source of specific molecular species of PC?

It has been speculated that a function of PE methylation may be the generation of polyunsaturated species of PC, given the highly unsaturated fatty acid composition of PE (10). As discussed above, purified PEMT displays a preference for unsaturated species of PE (151). Early *in vivo* experiments in rats were performed with argentation thin-layer chromatography, which will separate PC molecular species according to the total number of double bonds they contain. In these experiments, PC radiolabeled with [^{14}C]methionine generally

has the same fatty acid composition as PE, i.e. enriched in tetra- and hexaenoic fractions (249-252).Therefore, it appears that the molecular species specificity of PEMT is solely a reflection of the species of PE present. Similar results were seen when microsomal PE was used as a substrate for purified PEMT (151). In intact hepatocytes however, incubation with [*methyl*-^{3}H]methionine, followed by isolation and fractionation by high performance liquid chromatography of radiolabeled PC reveals a slight preference for 1-palmitoyl-2-docosahexaenoyl PC (151). In contrast, saturated and lightly unsaturated PCs are the primary product of the CDP-choline pathway (10,253). However, constant deacylation-reacylation of PC derived originally from ethanolamine occurs such that recently methylated PC is indistinguishable from bulk PC within 12 - 18 hours (143,151). When PE methylation is inhibited with 3-deazaadenosine in rat hepatocytes, no effect is seen on the molecular species distribution of either PC or PE (254). In the fetal guinea pig liver, PC is enriched in the 22:6(Δ3) fatty acid at the *sn-2* position. Unlike rats, guinea pigs express PEMT embryologically and show no preference for newly-synthesized over bulk PC from ethanolamine as a substrate for methylation (145). Initial experiments suggest that unlike the rat, PE methylation appears necessary for generating highly unsaturated PC. However, deacylation-reacylation is again observed, ensuring that PEMT-derived PC resembles bulk PC in fatty acid composition . These results suggest that it is unlikely that PE methylation is responsible for generating a specific pool of PC with a particular fatty acid composition. Nonetheless, PEMT may still be responsible for bringing highly unsaturated fatty acids into the total PC pool, at least in some species.

1.3.4(d) Is PEMT a tumour suppressor?

In several cases, an inverse correlation between PEMT levels and hepatocyte growth rates has been observed:

1) Around the time of birth in rats, the growth rate of livers decreases, while PEMT activity and PEMT2 gene expression increase (215).

2) Following partial hepatectomy, hepatocyte proliferation is stimulated, and PEMT activity and PEMT2 gene expression are inhibited (221).

3) Stimulation of non-neoplastic liver growth with lead nitrate results in a decrease in PEMT activity and PEMT2 gene expression (223).

4) Hepatoma-derived cultured cells, which grow profusely, do not contain PEMT activity nor PEMT2 gene expression (153).

5) When liver cancer is induced with carcinogens, PEMT activity and PEMT2 protein expression are greatly reduced at the very earliest stage of tumour development (Tessitore, L. Cui, Z. and Vance, D. E., unpublished results).

These correlations suggest that PEMT may play a role in the regulation of hepatocyte proliferation. Expression of PEMT2 in McArdle RH7777 cells, a rat hepatoma cell line, decreases the growth rate of the culture (255). The inhibition of growth is directly related to the enzymatic conversion of PE to PC, rather than some other function of PEMT2. This effect appears to be hepatoma-specific, since the expression of PEMT2 in non-hepatic CHO cells does not alter growth rates (234). This result suggests that PEMT expression alters growth rates, rather than growth rates altering PEMT expression. It is worth noting that this laboratory has had difficulty duplicating the inhibition of McArdle RH7777 growth with PEMT2 (D. Vance, unpublished results). Clearly, further investigation of this phenomenon is required.

1.3.5 PEMT controversies

1.3.5(a) How many enzymes?

The fact that PE methylation occurs in three distinct steps has led to speculation that more than one enzyme is responsible for catalyzing the complete pathway in mammals. The finding that two enzymes are involved in PE methylation in yeast has given reinforcement to this hypothesis (163). Axelrod and co-workers proposed that two enzymes are responsible for complete PE methylation to PC: Methyltransferase I catalyzes the methylation of PE to PMME, and Methyltransferase II catalyzes the methylation PMME to PDME and PC. This conclusion is the result of analysis of pH optimum, K_m for AdoMet and divalent cation requirement differences between the two methyltransferases in a wide variety of tissues, including liver (256), brain (257,258), adrenal glands (259), erythrocytes (260), colon (261), and pituitary gland (262). Most importantly, the notion that there are two methyltransferases was based on the rapid accumulation of radioactivity in PMME and PDME following the addition of radiolabeled methionine, in order to calculate a K_m value for AdoMet. However, Audubert and Vance argue correctly that this accumulation of labeled intermediates is merely a reflection of steady state conditions (263). They find that after one minute of incubation, the radioactivity in PMME and PDME remains constant. Formulas that account for this fact result in calculated kinetic parameters and pH optima nearly identical for all steps, making the two-enzyme model unlikely. One study found that treating microsomes with phospholipase C hydrolyzes PC, but not PMME or PDME (264,265). The author interpreted these results to mean that the initial methylations occur on the lumenal side of microsomes with one methyltransferase, followed by translocation of the lipid intermediate to the outer

leaflet and further methylation to PC with a second methyltransferase. However, no account is made for the possibility that PMME and PDME are not substrates for phospholipase C, or that PMME and PDME are resistant to hydrolysis because they are sequestered by binding to PEMT. The notion of two enzymes catalyzing different parts of the PE methylation pathway is separate from the identification of two isoforms of PEMT, PEMT1 and PEMT2, since both these isoforms catalyze all three steps (153).

In addition to the purification described above, others have claimed to have isolated PEMT from liver. Mato and co-workers described the purification of a 50 kDa protein from rat liver that they claim contains PEMT activity (266,267). This protein, which appears to be a homodimer of two 25 kDa subunits, has been used for further studies on the regulation of PEMT (213,268-270). However, there is little direct evidence that this protein catalyzes PE methylation. As well, another group claims to have purified a methyltransferase from mouse liver microsomes that will only methylate PMME and PDME, not PE (271). However, the activity of this methyltransferase is extremely low compared to the purified PEMT described by Ridgway and Vance (149).

1.3.5(b) Is PEMT expressed in extrahepatic tissues?

It is interesting to note that the discussion above refers to the study of PE methylation in tissues other than the liver. An initial tissue survey by Bremer and Greenberg using endogenous PE as a substrate reveals the highest level of PEMT activity in the liver of rats, with significantly lower, but still measurable, activity in testis, kidney and heart, and perhaps lung (133). No activity above background levels is observed in brain, spleen and intestine. A later tissue

survey confirms these results (153). Despite these low levels of activity and the apparent minimal contribution of PE methylation to PC synthesis, PEMT has been studied exhaustively in extrahepatic tissues. This work is best summarized in a review by Vance and Ridgway (272). Photoaffinity labeling with [*methyl*-3H]AdoMet of post-mitochondrial supernatants labels a protein with a similar molecular mass to purified PEMT in liver, but not in kidney, lung or heart (152). Using the antibody against the C-terminus of the rat PEMT2 as a probe for immunoblots, expression of the PEMT2 protein is only detected in the liver (153). The low levels of PEMT activity observed in other tissues may be explained by chemical, rather than enzymatic, methylation of PE. Such an activity is detectable, particularly at the alkaline pHs typically used in PEMT assays (273).

1.3.5(c) Does PE methylation play a role in intracellular signaling?

A significant factor leading investigators to study PE methylation in extrahepatic tissues was the hypothesis that PE methylation plays a role in intracellular signaling. Axelrod and co-workers observed a stimulation of [*methyl*-^{3}H]methionine incorporation into PC in rat reticulocyte ghosts, HeLa cells, rat mast cells and other cell lines treated with a variety of agonists (274,275). This response preceded other cellular events, including generation of cAMP, histamine, arachidonate and Ca^{++} release, chemotaxis and mitogenesis. Methylation inhibitors attenuated this response (274). The authors proposed that PE methylation alters the viscosity of the plasma membrane in the microenvironment surrounding the receptors involved, allowing specific interactions with other components of the signaling pathway (274,275).

After intensive investigation, the notion that PE methylation plays a role in intracellular signaling has been widely dismissed. For one, PEMT activity in many of these cell types is less than 0.1% of that found in the liver, in particular in plasma membrane fractions (276). The extremely low measured activities can be accounted for by something as trivial as impure reagents. Perhaps more importantly, other investigators have been unable to reproduce the results of Axelrod and co-workers (277-279). Instead, the well-characterized PI/DAG/Ca++/protein kinase C pathway is activated in response to agonists in different cell lines (278). Interestingly, AdoHcy, whose levels are increased by methylation inhibitors, inhibits signaling by this pathway in leukocytes (280). This phenomenon may explain some of the results observed by Axelrod *et al.*

1.4 Choline

1.4.1 Roles of choline

The notion that choline is an essential nutrient was first raised by Best and Huntsman in 1932, who noted that a choline-deficient diet in rats results in an accumulation of triglycerides in the liver, producing the so-called "fatty liver" (281). In 1955, Eagle discovered that choline is necessary for the survival of cultured cells (282). The average human takes in 7-10 mmol of choline per day, primarily in the form of PC, but also as free choline, sphingomyelin and other choline metabolites (283). Eggs and liver are the best dietary sources of choline. The metabolic fates of choline are outlined in Figure 1.4.

1.4.1(a) Choline as a precursor of PC

The incorporation of choline into PC is described in detail above. In tissues other than the liver and kidney, phosphorylation (the first step in the CDP-choline pathway) is the primary fate of intracellular choline (284). The rate of incorporation of choline into PC in liver has been measured at 0.25 µmol/mg wet tissue/min (75).

1.4.1(b) Choline incorporation into acetylcholine

In the nervous system, cholinergic neurons incorporate choline into the neurotransmitter acetylcholine, as well as into PC. Cholinergic nerve terminals are thought to contain a second high affinity choline transporter in addition to the low affinity transporter found in all tissues (285). This transporter's activity is tightly linked to acetylcholine synthesis. The reaction of choline and acetyl-

coenzyme A (acetyl-CoA) is catalyzed by the enzyme choline acetyltransferase (ChAT). Reciprocal regulation of this enzyme and choline kinase is observed upon treatment of neurons with retinoic acid, an inducer of cholinergic differentiation, and K^+ ions, which cause depolarization of neurons (286).

1.4.1(c) Choline contribution to the 1-carbon pool

The majority of choline in the liver and kidney is oxidized to choline aldehyde, and subsequently converted to betaine (287). The choline oxidase system is composed of two enzymes: choline dehydrogenase, a membrane-bound mitochondrial protein, and betaine aldehyde dehydrogenase, which is found in both the cytosol and mitochondria (284). Since the formation of betaine from choline is irreversible, the pathway serves to modulate choline levels.

Betaine serves as methyl group donor by reacting with homocysteine to form methionine and dimethylglycine, catalyzed by the enzyme betaine:homocysteine methyltransferase (288). Methionine is subsequently converted to *S*-adenosylmethionine by AdoMet synthetase, which is found in all tissues, although the liver and extrahepatic forms are encoded by separate genes in mammals (202). *S*-adenosylmethionine is the primary donor of methyl groups in biochemical reactions, methylating not only PE, but also proteins, nucleic acids and a wide variety of small molecules. In the kidney, betaine serves as an osmolyte (289).

1.4.2 Choline deficiency

As noted above, a lack of dietary choline results in the accumulation of hepatic triglycerides, producing a fatty liver. As well, the levels of hepatic PC are decreased, while the levels of hepatic PE are increased (232,290,291). Since then, the physiological effects of a diet deficient in choline have been studied extensively. It is worth noting that a choline-deficient diet is artificial; virtually all foods consumed by mammals contain at least some choline, usually as PC (284).

The accumulation of triglycerides in the liver begins very quickly once an animal begins feeding on a choline-deficient diet, with a fatty liver appearing within hours in rats (292). Diacylglycerol also accumulates in the fatty liver (293). Hepatocytes isolated from a choline-deficient rat and cultured without choline and methionine are impaired in their ability to synthesize PC, resulting in an increase in PE and a decrease in PC, as in the whole liver (232,294,295). This impairment of PC synthesis inhibits the formation and secretion of very low-density lipoproteins (VLDL), but not high- density lipoproteins (HDL) (239,296). Triglyceride, cholesterol and apoB-containing lipoprotein levels are reduced in the plasma of the choline-deficient rat (297,298). This result highlights the importance of PC biosynthesis in lipoprotein metabolism, and points to a cause for the fatty liver. Replenishment of cultured hepatocytes with choline, methionine or betaine plus homocysteine restores VLDL secretion (239,299). As well, a choline deficient diet results in kidney damage to rats and other species, particularly to weanlings (300). This effect may be related to betaine's function as an osmolyte. Finally, rats fed a choline-deficient diet suffer from a greater frequency of hepatocarcinogenesis, as well as increased sensitivity to carcinogens (301). Several hypotheses have been put forward as explanations for this phenomenon, including the accumulation of DAG and sustained signal

transduction through protein kinase C, undermethylation of DNA, and increased lipid peroxidation generating free radicals (283).

Despite the importance of PC biosynthesis, choline deficiency is not lethal. Therefore, animals are able to compensate for the loss of choline. Since PEMT is the only endogenous source of choline, its regulation in response to choline deficiency has been examined. In the livers of three-day choline-deficient rats, the conversion of PE to PC is decreased approximately two-fold, which may be the cause of the elevated hepatic PE levels observed in these animals (290,296,302). In contrast, PEMT activity in microsomes from three-day choline-deficient rat livers is increased two-fold compared to choline-supplemented rat livers, due evidently to the stimulatory effects of the elevation of hepatic PE levels (232,290,303). The apparent difference between the *in vivo* and *in vitro* PEMT activities is most likely due to a decrease in the AdoMet/AdoHcy ratio in hepatocytes, another effect of choline deficiency (304). Indeed, supplementation of choline-deficient hepatocytes with methionine stimulates incorporation of radiolabeled ethanolamine into PC, restores PC and PE levels, and restarts VLDL secretion (239,305). After 6 weeks a five-fold increase in PEMT2 protein levels and a concomitant increase in PEMT2 mRNA levels accompany a two-fold elevation of PEMT activity, while PC and PE levels return to the level observed in choline-supplemented livers (233). Therefore, the elevation of *in vitro* PEMT activity in response to choline deficiency appears to be biphasic. In contrast, the enzymes of the CDP-choline and CDP-ethanolamine pathway are not stimulated in the choline-deficient liver, except for CT, which translocates to the activated membrane-bound form (303,306). Since CT is the regulatory enzyme for the CDP-choline pathway, its activation may be an adaptation designed to ensure that choline released from PC

turnover is efficiently re-incorporated. These results suggest that PC derived from PE methylation, and the choline thus generated, is able to compensate for the lack of dietary choline in the whole animal.

1.5 Thesis Objective

In all eukaryotic cells, PC is synthesized from choline via the CDP-choline pathway. The mammalian liver contains a second pathway for the biosynthesis of PC: the methylation of PE. The three sequential transmethylations are catalyzed by PEMT, using AdoMet as a methyl group donor. The ultimate goal of this thesis is to answer the question: "Why has the liver retained PEMT2 and thus PE methylation as a source of PC, when all other tissues can generate the PC they need solely through the CDP-choline pathway?" We hypothesize that PEMT-derived PC serves a function other than contributing to bulk hepatic phospholipids. As a way of determining this function, we chose the "knockout" mouse model. By constructing a mouse in which the PEMT2 gene is disrupted and analyzing the resulting phenotype, we hope to gain insight into the role of PE methylation in the whole animal. In particular, by feeding the mice a choline-deficient diet, we hope to ascertain the importance of PEMT in the endogenous synthesis of choline. Along the way, we intend to learn the structure and chromosomal localization of the PEMT2 gene, and discover the relationship between the origins of the various PEMTs, such as whether PEMT1 and PEMT2 are encoded by the same gene.

PEMT2 gene expression occurs solely in the liver, in fully differentiated hepatocytes. Using the knowledge gained from the characterization of the mouse PEMT2 gene, and in particular its 5′ end, we will attempt to explain the molecular basis of this pattern of expression, in particular the role of the transcription factor C/EBPα. Finally, PEMT2 has been implicated as a potential tumour suppressor. This thesis will outline the initial steps in determining the

importance of PEMT in human hepatocarcinogenesis. These include cDNA and genomic cloning of the human PEMT2, and its chromosomal localization.

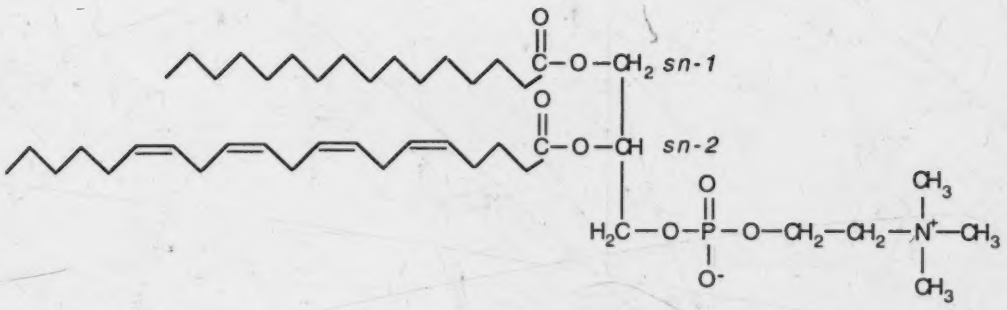


Figure 1.1 **Structure of phosphatidylcholine**

The molecular structure of a typical molecule of PC (1-palmitoyl-2-arachidonyl-phosphatidylcholine is shown.

Figure 1.2 **The CDP-choline pathway for the biosynthesis of PC**

The pathway for the synthesis of PC from choline is shown. Choline is transported into the cell and phosphorylated by choline kinase. Phosphocholine is subsequently activated by its reaction with CTP to form CDP-choline. This is the rate-limiting step in the pathway, catalyzed by CTP:phosphocholine cytidylyltransferase. Finally, phosphocholine is transferred from CDP-choline to DAG, forming PC. This last step is catalyzed by choline phosphotransferase.

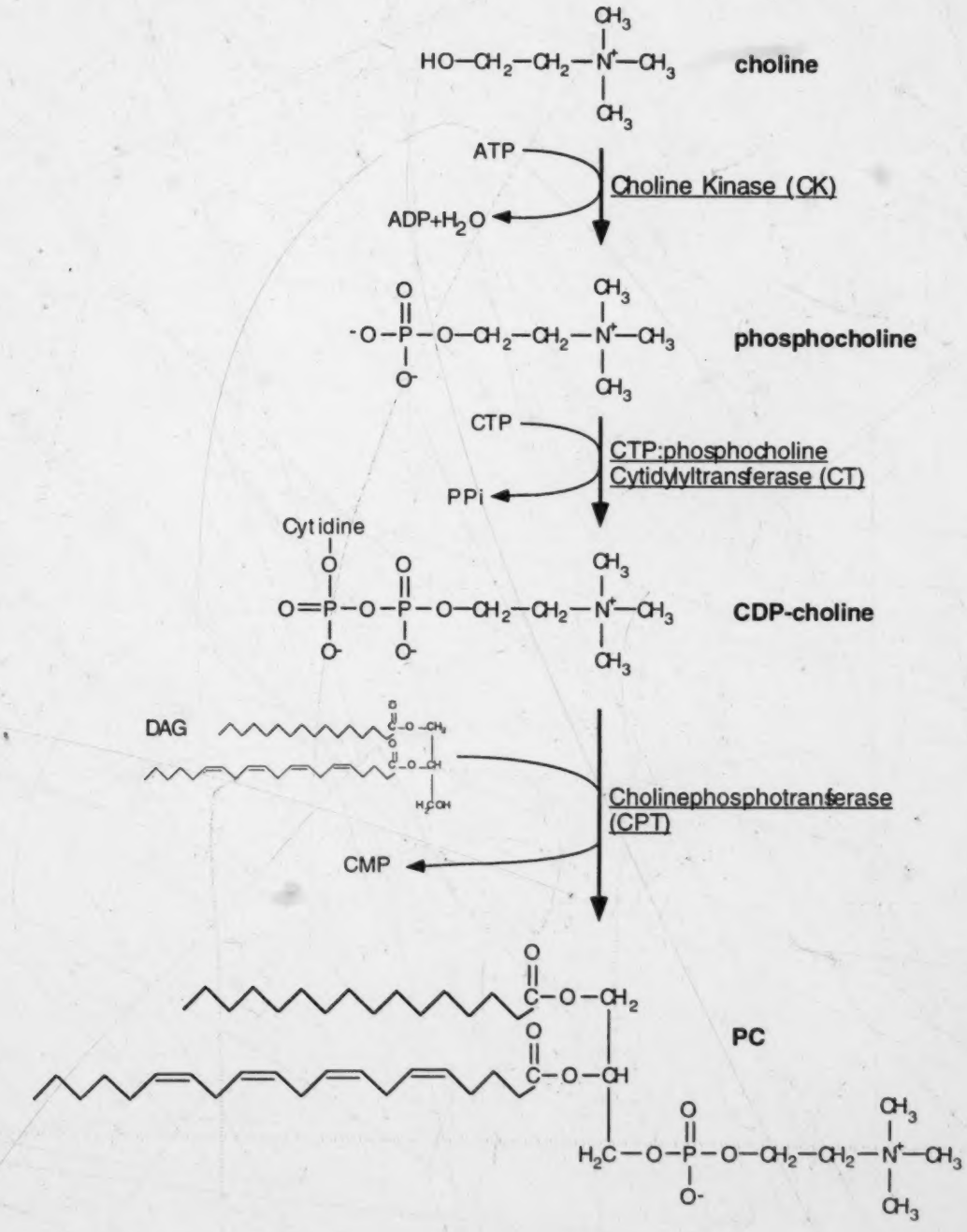

choline
ATP
Choline Kinase (CK)
ADP+H2O
phosphocholine
CTP
CTP:phosphocholine
Cytidylyltransferase (CT)
PPi
Cytidine
CDP-choline
DAG
Cholinephosphotransferase
(CPT)
CMP
PC

Figure 1.3 **The PE methylation pathway for the biosynthesis of PC**

Phosphatidylethanolamine (PE) is methylated three times, with *S*-adenosylmethionine (AdoMet) as the methyl group donor. All three steps are catalyzed by the enzyme phosphatidylethanolamine *N*-methyltransferase (PEMT). In mammals, this pathway occurs to a significant degree only in the liver.

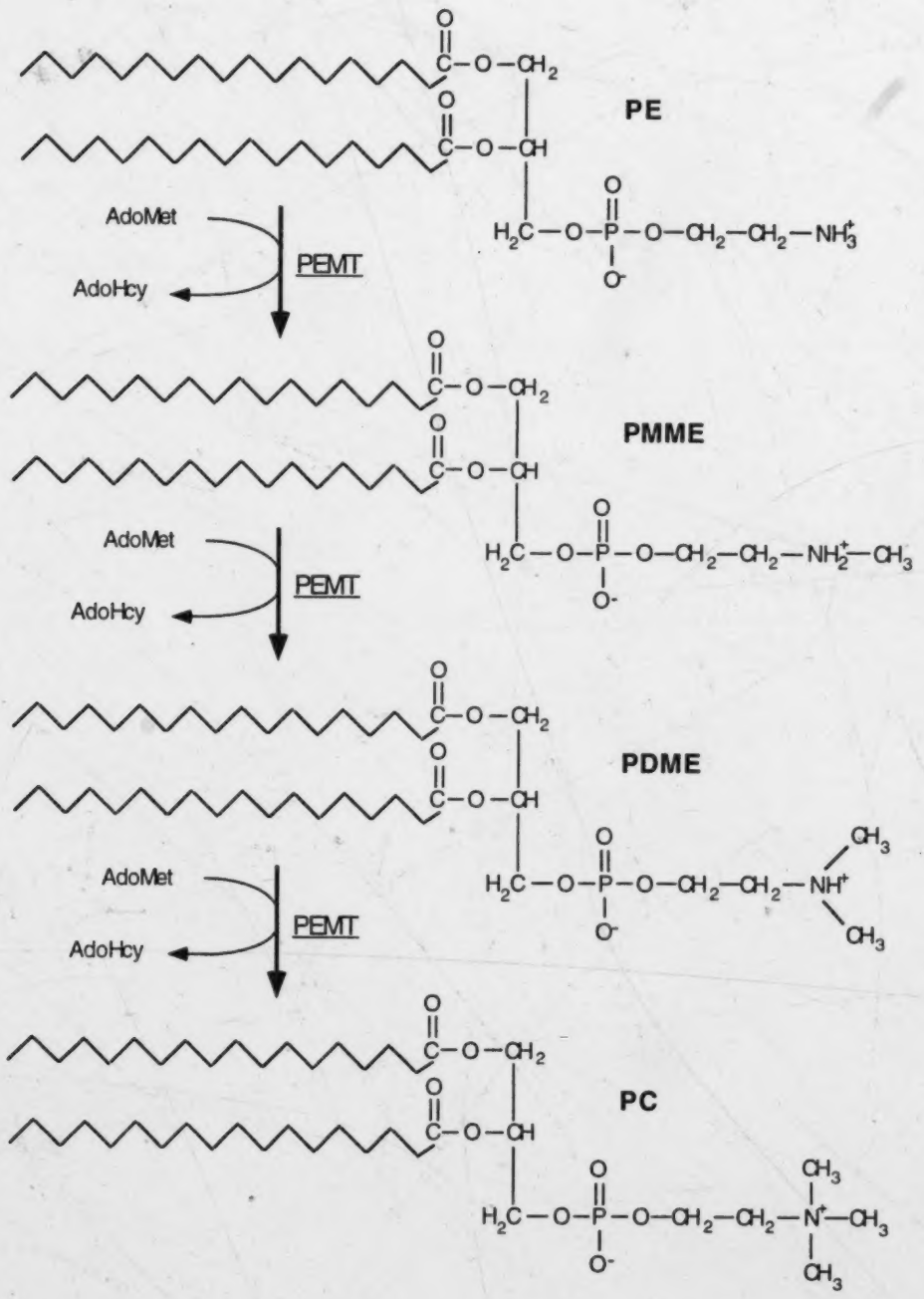
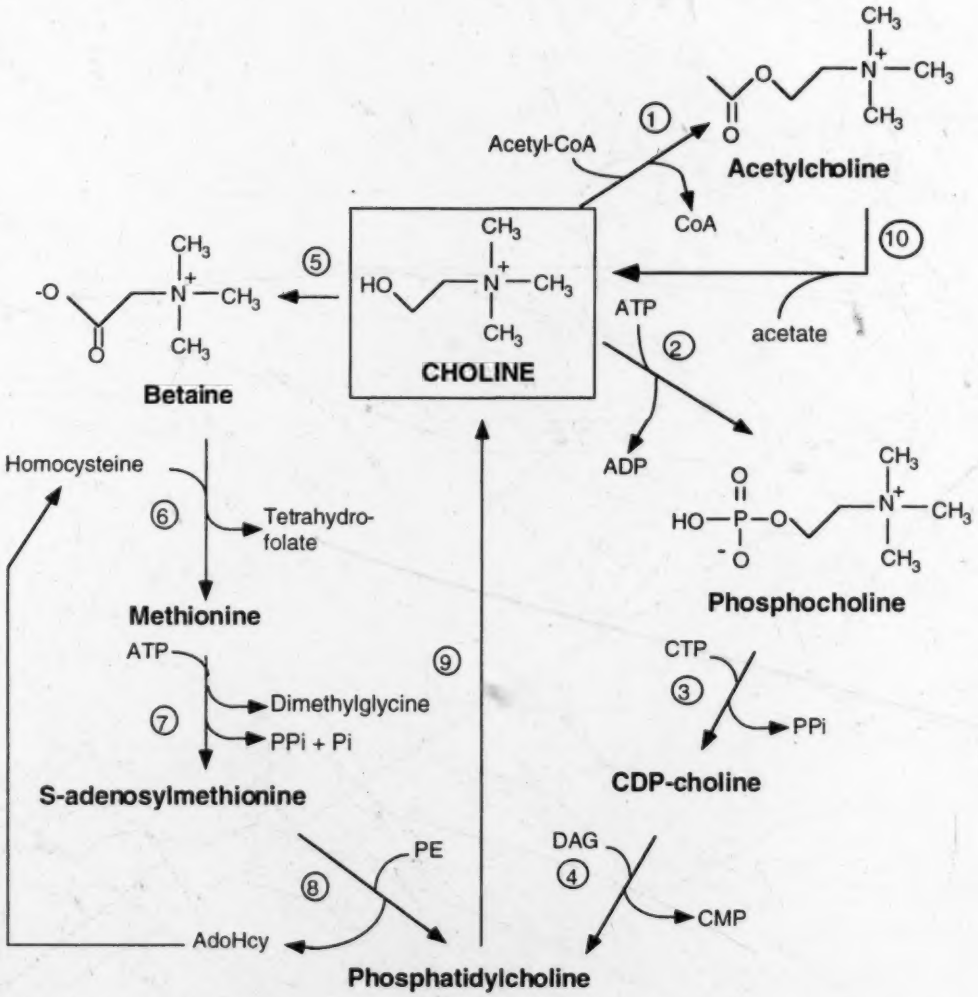

PE
AdoMet
AdoHcy
PEMT
PMME
AdoMet
AdoHcy
PEMT
PDME
AdoMet
AdoHcy
PEMT
PC

Figure 1.4 **The metabolic fates of choline**

The possible metabolic fates of choline transported into the cell are shown. Numbers represent enzymes involved, with mammalian tissue distributions in parentheses:

1) Choline acetyltransferase (cholinergic neurons)
2) Choline kinase (all tissues)
3) CTP:phosphocholine cytidylyltransferase (all tissues)
4) Choline phosphotransferase (all tissues)
5) Choline dehydrogenase (liver and kidney)
   + Betaine aldehyde dehydrogenase (liver and kidney)
6) Betaine:homocysteine methyltransferase (liver)
7) *S*-adenosylmethionine synthetase (all tissues)
8) Phosphatidylethanolamine *N*-methyltransferase (liver)
9) Phospholipase D (all tissues)
10) Acetylcholinesterase (cholinergic neurons)


Acetylcholine
Acetyl-CoA
CoA
CHOLINE
Betaine
acetate
ATP
ADP
Phosphocholine
Homocysteine
Tetrahydro-
folate
Methionine
ATP
Dimethylglycine
PPi + Pi
S-adenosylmethionine
CTP
PPi
CDP-choline
PE
AdoHcy
DAG
CMP
Phosphatidylcholine

References

1. Ansell, G. B., and Spanner, S. (1982) in *Phospholipids* (Hawthorne, J. N., and Ansell, G. B., eds), pp. 1-49, Elsevier, Amsterdam
2. Gobley, M. (1850) *J. Pharm. Chim.* **17,** 401-407
3. Diaconow, C. (1868) *Zbl. med. Wiss.* **2,** 434-435
4. Strecker, A. (1868) *Ann. Chem. Pharm.* **148,** 77-90
5. Baer, E., and Kates, M. (1950) *Journal of the American Chemical Society* **72,** 942-949
6. Cullis, P. R., Fenske, D. B., and Hope, M. J. (1996) in *Biochemistry of Lipids, Lipoproteins and Memebranes* (Vance, D. E., and Vance, J. E., eds), pp. 1-33, Elsevier, Amsterdam
7. Tattrie, N. H. (1959) *Journal of Lipid Research* **1,** 60-65
8. Hanahan, D. J., Brockerhoff, H., and Barron, E. J. (1960) *Journal of Biological Chemistry* **235,** 1917-1923
9. de Haas, G. H., Daemen, F. J. M., and van Deenen, L. L. M. (1962) *Biochimica et Biophysica Acta* **65,** 260-270
10. Holub, B. J., and Kuksis, A. (1971) *Canadian Journal of Biochemistry* **49**(12), 1347-56
11. Mueller, H. W., O'Flaherty, J. T., and Wykle, R. L. (1982) *Lipids* **17**(2), 72-77
12. Ojima-Uchiyama, A., Masuzawa, Y., Sugiura, T., Waku, K., Saito, H., Yui, Y., and Tomioka, H. (1988) *Lipids* **23**(8), 815-817
13. Arthur, G., Mock, T., Zaborniak, C., and Choy, P. C. (1985) *Lipids* **20**(10), 693-698
14. Thompson, G. A. (1992) *The Regulation of Membrane Lipid Metabolism*, 3rd Ed., CRC Press, Boca Raton

15. Presti, F. T. (1985) in *Membrane Fluidity in Biology* (Aloia, R. C., and Boggs, J. M., eds), pp. 97-146, Academic Press, New York

16. Singer, S. J., and Nicolson, G. L. (1972) *Science* **175**(23), 720-731

17. Chapman, M. J. (1986) *Methods in Enzymology* **128,** 70-143

18. Nelson, G. J. (1967) *Lipids* **2,** 323-328

19. Coleman, R. (1987) *Biochemical Journal* **244**(2), 249-261

20. Van Nieuwkerk, C. M. J., Oude Elferink, R. P. J., Groen, A. K., Ottenhoff, R., Tytgat, G. N. J., Dingemans, K. P., Van den Bergh Weerman, M. A., an d Offerhaus, G. J. A. (1996) *Gastroentoerology* **111,** 165-171

21. Smit, J. J. M., Schinkel, A. H., Oude Elferink, R. P. J., Groen, A. K., Wagenaar, E., van Deemter, L., Mol, C. A. A. M., Ottenhoff, R., van der Lugt, N. M. T., van Roon, M. A., van der Valk, M. A., Offerhaus, G. J. A., Berns, A. J. M., and Borst, P. (1993) *Cell* **75,** 451-462

22. Beukeveld, G. J., In't Veld, G., Havinga, R., Groen, A. K., Wolthers, B. G., and Kuipers, F. (1996) *Journal of Hepatology* **24**(3), 343-352

23. Minich, D. M., Vonk, R. J., and Verkade, H. J. (1997) *Journal of Lipid Research* **38,** 1709-1721

24. Tso, P., Balint, J. A., and Simmonds, W. J. (1977) *Gastroenterology* **73,** 1362-1367

25. Hay, D. W., and Carey, M. C. (1990) *Hepatology* **12**(3 Pt 2), 6S-14S; discussion 14S-16S

26. Ruetz, S., and Gros, P. (1994) *Cell* **77,** 1071-1081

27. Possmayer, F. (1989) in *Phosphatidylcholine Metabolism* (Vance, D. E., ed), pp. 205-223, CRC Press, Boca Raton

28. Smith, W. L., and Fitzpatrick, F. A. (1996) in *Biochemistry of Lipids, Lipoproteins and Membranes* (Vance, D. E., and Vance, J. E., eds), pp. 283-308, Elsevier, Amsterdam

29. Asaoka, Y., Oka, M., Yoshida, K., Sasaki, Y., and Nishizuka, Y. (1992) *Proceedings of the National Academy of Sciences ,USA* **89**(14), 6447-6451

30. Exton, J. H. (1990) *Journal of Biological Chemistry* **265**(1), 1-4

31. Exton, J. H. (1994) *Biochimica et Biophysica Acta* **1212**(1), 26-42

32. Billah, M. M., and Anthes, J. C. (1990) *Biochemical Journal* **269**(2), 281-291

33. Shukla, S. D., and Halenda, S. P. (1991) *Life Sciences* **48**(9), 851-866

34. Cockcroft, S. (1992) *Biochimica et Biophysica Acta* **1113**(2), 135-160

35. Pelech, S. L., and Vance, D. E. (1989) *Trends in Biochemical Science* **14**, 28-30

36. Zhang, Y., and Kolesnick, R. (1995) *Endocrinology* **136**(10), 4157-4160

37. Kolesnick, R., and Fuks, Z. (1995) *Journal of Experimental Medicine* **181**(6), 1949-1952

38. Snyder, F. (1995) *Biochimica et Biophysica Acta* **1254**(3), 231-249

39. Chao, W., and Olson, M. S. (1993) *Biochemical Journal* **292**(Pt 3), 617-629

40. van Corven, E. J., Groenink, A., Jalink, K., Eichholtz, T., and Moolenaar, W. H. (1989) *Cell* **59**(1), 45-54

41. van Corven, E. J., van Rijswijk, A., Jalink, K., van der Bend, R. L., van Blitterswijk, W. J., and Moolenaar, W. H. (1992) *Biochemical Journal* **281**(Pt 1), 163-169

42. Moolenaar, W. H. (1991) *Advances in Cancer Research* **57**, 87-102

43. Zhang, H., Desai, N. N., Murphey, J. M., and Spiegel, S. (1990) *Journal of Biological Chemistry* **265**(34), 21309-21316

44. Zhang, H., Desai, N. N., Olivera, A., Seki, T., Brooker, G., and Spiegel, S. (1991) *Journal of Cell Biology* **114**(1), 155-167

45. Desai, N. N., Zhang, H., Olivera, A., Mattie, M. E., and Spiegel, S. (1992) *Journal of Biological Chemistry* **267**(32), 23122-23128

46. Esko, J. D., Wermuth, M. M., and Raetz, C. R. (1981) *Journal of Biological Chemistry* **256**(14), 7388-7393

47. Cui, Z., Houweling, M., Chen, M. H., Record, M., Chap, H., Vance, D. E., and Terce, F. (1996) *Journal of Biological Chemistry* **271**(25), 14668-14671

48. Kennedy, E. (1989) in *Phosphatidylcholine Metabolism* (Vance, D. E., ed), pp. 1-8, CRC Press, Boca Raton

49. Jackowski, S. (1994) *Journal of Biological Chemistry* **269**(5), 3858-3867

50. Cottrell, S. F., Getz, G. S., and Rabinowitz, M. (1981) *Journal of Biological Chemistry* **256**(21), 10973-10978

51. Jackowski, S. (1996) *Journal of Biological Chemistry* **271**(34), 20219-20222

52. Terce, F., Brun, H., and Vance, D. E. (1994) *Journal of Lipid Research* **35**(12), 2130-2142

53. George, T. P., Morash, S. C., Cook, H. W., Byers, D. M., Palmer, F. B., and Spence, M. W. (1989) *Biochimica et Biophysica Acta* **1004**(3), 283-291

54. George, T. P., Cook, H. W., Byers, D. M., Palmer, F. B., and Spence, M. W. (1991) *Journal of Biological Chemistry* **266**(19), 12419-12423

55. Tijburg, L. B., Nishimaki-Mogami, T., and Vance, D. E. (1991) *Biochimica et Biophysica Acta* **1085**(2), 167-177

56. Tronchere, H., Planat, V., Record, M., Terce, F., Ribbes, G., and Chap, H. (1995) *Journal of Biological Chemistry* **270**(22), 13138-13146

57. Walkey, C. J., Kalmar, G. B., and Cornell, R. B. (1994) *Journal of Biological Chemistry* **269**(8), 5742-5749

58. Wittenberg, J., and Kornberg, A. (1953) *Journal of Biological Chemistry* **202**, 431-444

59. Ishidate, K., Nakagomi, K., and Nakazawa, Y. (1984) *Journal of Biological Chemistry* **259**(23), 14706-14710

60. Porter, T. J., and Kent, C. (1990) *Journal of Biological Chemistry* **265**(1), 414-422

61. Uchida, T., and Yamashita, S. (1990) *Biochimica et Biophysica Acta* **1043**(3), 281-288

62. Uchida, T. (1994) *Journal of Biochemistry* **116**(6), 1241-1250

63. Tadokoro, K., Ishidate, K., and Nakazawa, Y. (1985) *Biochimica et Biophysica Acta* **835**(3), 501-513

64. Uchida, T., and Yamashita, S. (1992) *Journal of Biological Chemistry* **267**(14), 10156-10162

65. Hosaka, K., Kodaki, T., and Yamashita, S. (1989) *Journal of Biological Chemistry* **264**(4), 2053-2059

66. Hosaka, K., Tanaka, S., Nikawa, J., and Yamashita, S. (1992) *FEBS Letters* **304**(2-3), 229-232

67. Uchida, T. (1994) *Journal of Biochemistry* **116**(3), 508-518

68. Uchida, T. (1996) *Biochimica et Biophysica Acta* **1304**(2), 89-104

69. Warden, C. H., and Friedkin, M. (1984) *Biochimica et Biophysica Acta* **792**(3), 270-280

70. Cuadrado, A., Carnero, A., Dolfi, F., Jimenez, B., and Lacal, J. C. (1993) *Oncogene* **8**(11), 2959-2968

71. Warden, C. H., and Friedkin, M. (1985) *Journal of Biological Chemistry* **260**(10), 6006-6011

72. Macara, I. G. (1989) *Molecular & Cellular Biology* **9**(1), 325-328

73. Teegarden, D., Taparowsky, E. J., and Kent, C. (1990) *Journal of Biological Chemistry* **265**(11), 6042-7

74. Lacal, J. C. (1990) *Molecular & Cellular Biology* **10**(1), 333-340

75. Sundler, R., Arvidson, G., and Akesson, B. (1972) *Biochimica et Biophysica Acta* **280**(4), 559-568

76. Vance, D. E., and Paddon, H. B. (1978) *Proceedings of the Canadian Federation of Biological Societies* **21,** 181

77. Vance, D. E., and Choy, P. C. (1979) *Trends in Biochemical Science* **4,** 145-148

78. Vance, D. E., and Pelech, S. L. (1984) *Trends in Biochemical Science* **9,** 17-20

79. Pelech, S. L., and Vance, D. E. (1984) *Biochimica et Biophysica Acta* **779**(2), 217-251

80. Tronchere, H., Record, M., Terce, F., and Chap, H. (1994) *Biochimica et Biophysica Acta* **1212**(2), 137-151

81. Kent, C. (1995) *Annual Review of Biochemistry* **64,** 315-343

82. Houweling, M., Jamil, H., Hatch, G. M., and Vance, D. E. (1994) *Journal of Biological Chemistry* **269**(10), 7544-7551

83. Utal, A. K., Jamil, H., and Vance, D. E. (1991) *Journal of Biological Chemistry* **266**(35), 24084-24091

84. Cornell, R., and Vance, D. E. (1987) *Biochimica et Biophysica Acta* **919**(1), 26-36

85. Jamil, H., Yao, Z. M., and Vance, D. E. (1990) *Journal of Biological Chemistry* **265**(8), 4332-4339

86. Cui, Z., Houweling, M., and Vance, D. E. (1995) *Biochemical Journal* **312**(Pt 3), 939-945

87. Jamil, H., Hatch, G. M., and Vance, D. E. (1993) *Biochemical Journal* **291**(Pt 2), 419-427

88. Weinhold, P. A., Rounsifer, M. E., and Feldman, D. A. (1986) *Journal of Biological Chemistry* **261**(11), 5104-5110

89. Feldman, D. A., and Weinhold, P. A. (1987) *Journal of Biological Chemistry* **262**(19), 9075-9081

90. Sanghera, J. S., and Vance, D. E. (1989) *Journal of Biological Chemistry* **264**(2), 1215-1223

91. Cornell, R. B. (1989) *Journal of Biological Chemistry* **264**, 9077-9082

92. Kalmar, G. B., Kay, R. J., Lachance, A., Aebersold, R., and Cornell, R. B. (1990) *Proceedings of the National Academy of Sciences of the United States of America* **87**(16), 6029-6033

93. Hogan, M., Zimmermann, L. J., Wang, J., Kuliszewski, M., Liu, J., and Post, M. (1994) *American Journal of Physiology* **267**(1 Pt 1), L25-32

94. Rutherford, M. S., Rock, C. O., Jenkins, N. A., Gilbert, D. J., Tessner, T. G., Copeland, N. G., and Jackowski, S. (1993) *Genomics* **18**(3), 698-701

95. Sweitzer, T. D., and Kent, C. (1994) *Archives of Biochemistry & Biophysics* **311**(1), 107-116

96. Kalmar, G. B., Kay, R. J., LaChance, A. C., and Cornell, R. B. (1994) *Biochimica et Biophysica Acta* **1219**(2), 328-334

97. Nikawa, J., Yonemura, K., and Yamashita, S. (1983) *European Journal of Biochemistry* **131**(1), 223-229

98. Tsukagoshi, Y., Nikawa, J., and Yamashita, S. (1987) *European Journal of Biochemistry* **169**(3), 477-486

99. Tang, W., Keesler, G. A., and Tabas, I. (1997) *Journal of Biological Chemistry* **272**(20), 13146-13151

100. Pelech, S. L., Cook, H. W., Paddon, H. B., and Vance, D. E. (1984) *Biochimica et Biophysica Acta* **795**(3), 433-440

101. Terce, F., Record, M., Tronchere, H., Ribbes, G., and Chap, H. (1991) *Biochimica et Biophysica Acta* **1084**(1), 69-77

102. Vance, J. E., and Vance, D. E. (1988) *Journal of Biological Chemistry* **263**(12), 5898-5909

103. Cornell, R. B., and MacLennan, D. H. (1985) *Biochimica et Biophysica Acta* **821**, 97-105

104. Morand, J. N., and Kent, C. (1989) *Journal of Biological Chemistry* **264**(23), 13785-13792

105. Watkins, J. D., and Kent, C. (1992) *Journal of Biological Chemistry* **267**(8), 5686-5692

106. Wang, Y., Sweitzer, T. D., Weinhold, P. A., and Kent, C. (1993) *Journal of Biological Chemistry* **268**(8), 5899-5904

107. Wang, Y., MacDonald, J. I., and Kent, C. (1993) *Journal of Biological Chemistry* **268**(8), 5512-5518

108. Wang, Y., MacDonald, J. I., and Kent, C. (1995) *Journal of Biological Chemistry* **270**(1), 354-360

109. Houweling, M., Cui, Z., Anfuso, C. D., Bussiere, M., Chen, M. H., and Vance, D. E. (1996) *European Journal of Cell Biology* **69**(1), 55-63

110. Tsukagoshi, Y., Nikawa, J., Hosaka, K., and Yamashita, S. (1991) *Journal of Bacteriology* **173**(6), 2134-2136

111. Craig, L., Johnson, J. E., and Cornell, R. B. (1994) *Journal of Biological Chemistry* **269**(5), 3311-3317

112. Cornell, R. B., Kalmar, G. B., Kay, R. J., Johnson, M. A., Sanghera, J. S., and Pelech, S. L. (1995) *Biochemical Journal* **310**(Pt 2), 699-708

113. Yang, J., Wang, J., Tseu, I., Kuliszewski, M., Lee, M., and Post, M. (1997) *Biochemical Journal* **325**, 29-38

114. Wang, Y., and Kent, C. (1995) *Journal of Biological Chemistry* **270**(30), 17843-17849

115. Yang, W., Boggs, K. P., and Jackowski, S. (1995) *Journal of Biological Chemistry* **270**(41), 23951-23957

116. Tessner, T. G., Rock, C. O., Kalmar, G. B., Cornell, R. B., and Jackowski, S. (1991) *Journal of Biological Chemistry* **266**(25), 16261-16264

117. Houweling, M., Tijburg, L. B., Vaartjes, W. J., Batenburg, J. J., Kalmar, G. B., Cornell, R. B., and Van Golde, L. M. (1993) *European Journal of Biochemistry* **214**(3), 927-933

118. Hogan, M., Kuliszewski, M., Lee, W., and Post, M. (1996) *Biochemical Journal* **314**(Pt 3), 799-803

119. Ishidate, K., and Weinhold, P. A. (1981) *Biochimica et Biophysica Acta* **664**, 133-147

120. Van Heusden, G. P., Ruestow, B., Van der Mast, M. A., and Van den Bosch, H. (1981) *Biochimica et Biophysica Acta* **666**(3), 313-321

121. Van Heusden, G. P., and Van den Bosch, H. (1982) *Biochimica et Biophysica Acta* **711**(2), 361-368

122. Ide, H., and Weinhold, P. A. (1982) *Journal of Biological Chemistry* **257**(24), 14926-14931

123. Rustow, B., Kunze, D., Rabe, H., and Reichmann, G. (1985) *Biochimica et Biophysica Acta* **835**(3), 465-476

124. Morimoto, K., and Kanoh, H. (1978) *Journal of Biological Chemistry* **253**(14), 5056-5060

125. Van Golde, L. M. G., Raben, J., Batenburg, J. J., Fleischer, B., Zambrano, F., and Fleischer, S. (1974) *Biochimica et Biophysica Acta* **360**, 179-192

126. Jelsema, C. L., and Morre, D. J. (1978) *Journal of Biological Chemistry* **253**(21), 7960-7971

127. Higgins, J. A., and Fieldsend, J. K. (1987) *Journal of Lipid Research* **28**(3), 268-278

128. Hjelmstad, R. H., and Bell, R. M. (1987) *Journal of Biological Chemistry* **262**(8), 3909-3917

129. Du Vigneaud, V., Cohn, M., Chandler, J. P., Schenk, J. R., and Simmonds, S. (1941) *Journal of Biological Chemistry* **140,** 625-641

130. Stetten, D. (1941) *Journal of Biological Chemistry* **138,** 437-438

131. Bremer, J., and Greenberg, D. M. (1960) *Biochimica et Biophysica Acta* **37,** 173-175

132. Bremer, J., Figard, P. H., and Greenberg, D. M. (1960) *Biochimica et Biophysica Acta* **43,** 477-488

133. Bremer, J., and Greenberg, D. M. (1961) *Biochimica et Biophysisca Acta* **46,** 205-216

134. Bremer, J., and Greenberg, D. M. (1959) *Biochimica et Biophysica Acta* **35,** 28-29

135. Katyal, S. L., and Lombardi, B. (1974) *Lipids* **9**(2), 81-85

136. Schneider, W. J., and Vance, D. E. (1979) *Journal of Biological Chemistry* **254**(10), 3886-3891

137. Audubert, F., and Vance, D. E. (1984) *Biochimica et Biophysica Acta* **792**(3), 359-362

138. Bell, R. M., Ballas, L. M., and Coleman, R. A. (1981) *Journal of Lipid Research* **22,** 391-403

139. Vance, D. E., Choy, P. C., Farren, S. B., Lim, P. H., and Schneider, W. J. (1977) *Nature* **270**(5634), 268-269

140. Sundler, R., and Akesson, B. (1975) *Journal of Biological Chemistry* **250**(9), 3359-3367

141. Wise, E. M. J., and Elwyn, D. (1965) *Journal of Biological Chemistry* **240**(4), 1537-1547

142. Bjornstad, P., and Bremer, J. (1966) *Journal of Lipid Research* **7,** 38-45

143. Samborski, R. W., Ridgway, N. D., and Vance, D. E. (1990) *Journal of Biological Chemistry* **265**(30), 18322-18329

144. Samborski, R. W., and Vance, D. E. (1993) *Biochimica et Biophysica Acta* **1167**(1), 15-21

145. Burdge, G. C., Kelly, F. J., and Postle, A. D. (1993) *Biochemical Journal* **290**(Pt 1), 67-73

146. Vance, J. E., and Vance, D. E. (1986) *Journal of Biological Chemistry* **261**(10), 4486-4491

147. Hazel, J. R. (1990) *American Journal of Physiology* **258**(6 Pt 2), R1495-1501

148. Ridgway, N. D. (1989) in *Phosphatidylcholine metabolism* (Vance, D. E., ed), pp. 103-120, CRC Press, Boca Raton

149. Ridgway, N. D., and Vance, D. E. (1987) *Journal of Biological Chemistry* **262**(35), 17231-17239

150. Ridgway, N. D., and Vance, D. E. (1988) *Journal of Biological Chemistry* **263**(32), 16864-16871

151. Ridgway, N. D., and Vance, D. E. (1988) *Journal of Biological Chemistry* **263**(32), 16856-16863

152. Ridgway, N. D. (1988) Thesis in Depratment of Biochemistry, pp. 163, University of British Columbia, Vancouver, B. C.

153. Cui, Z., Vance, J. E., Chen, M. H., Voelker, D. R., and Vance, D. E. (1993) *Journal of Biological Chemistry* **268**(22), 16655-16663

154. Kodaki, T., and Yamashita, S. (1987) *Journal of Biological Chemistry* **262**(32), 15428-15435

155. Vance, J. E. (1990) *Journal of Biological Chemistry* **265**(13), 7248-56

156. Arondel, V., Benning, C., and Somerville, C. R. (1993) *Journal of Biological Chemistry* **268**(21), 16002-16008

157. Cain, B. D., Donohue, T. J., Shepherd, W. D., and Kaplan, S. (1984) *Journal of Biological Chemistry* **259**(2), 942-948

158. Kaneshiro, T., and Law, J. H. (1964) *Journal of Biological Chemistry* **239**(6), 1705-1703

159. Johnston, N. C., and Goldfine, H. (1983) *Journal of General Microbiology* **129**, 1075-1081

160. Tahara, Y., Ogawa, Y., Sakakibara, T., and Yamada, Y. (1986) *Agricultural Biological Chemistry* **50**, 257-259

161. Wilkinson, S. G. (1988) in *Microbial Lipids* (C., R., and Wilkinson, S. G., eds) Vol. 1, pp. 299-488, 2 vols., Academic Press, London

162. Goldfine, H. (1984) *Journal of Lipid Research* **25**, 1501-1507

163. Henry, S. A., and Patton-Vogt, J. L. (1997) *Progress in Nucleic Acids Research and Molecular Biology*, in press

164. Waechter, C. J., Steiner, M. R., and Lester, R. L. (1969) *Journal of Biological Chemistry* **244**(12), 3419-3422

165. Atkinson, K. D., Jensen, B., Kolat, A. I., Storm, E. M., Henry, S. A., and Fogel, S. (1980) *Journal of Bacteriology* **141**(2), 558-564

166. Patton-Vogt, J. L., Griac, P., Sreenivas, A., Bruno, V., Dowd, S., Swede, M. J., and Henry, S. A. (1997) *Journal of Biological Chemistry* **272**(33), 20873-20883

167. Greenberg, M. I., Reiner, B., and Henry, S. A. (1981) *Genetics* **100**, 19-33

168. Greenberg, M. L., Klig, L. S., Letts, V. A., Loewy, B. S., and Henry, S. A. (1983) *Journal of Bacteriology* **153**(2), 791-799

169. Summers, E. F., Letts, V. A., McGraw, P., and Henry, S. A. (1988) *Genetics* **120**(4), 909-922

170. Kodaki, T., and Yamashita, S. (1989) *European Journal of Biochemistry* **185**(2), 243-251

171. Preitschopf, W., Luckl, H., Summers, E., Henry, S. A., Paltauf, F., and Kohlwein, S. D. (1993) *Current Genetics* **23**, 95-101

172. Fernandez, S., Homann, M. J., Henry, S. A., and Carman, G. M. (1986) *Journal of Bacteriology* **166,** 779-786

173. Hill, J. E., Chung, C., McGraw, P., Summers, E., and Henry, S. A. (1990) in *Biochemistry of cell walls and membranes in fungi* (Kuhn, P. J., Trinci, A. P. J., Jung, M. J., Goosey, M. W., and Copping, L. G., eds), pp. 246-260, Springer-Verlag, Amsterdam

174. Kanipes, M. I. (1997) Thesis in Department of Biological Sciences, pp. 147, Carnegie Mellon University, Pittsburgh, Pennsylvania

175. Hall, M. O., and Nyc, J. F. (1959) *Journal of the American Chemical Society* **81,** 2275

176. Crocken, B. J., and Nyc, J. F. (1964) *Journal of Biological Chemistry* **239**(6), 1727-1730

177. Scarborough, G. A., and Nyc, J. F. (1967) *Journal of Biological Chemistry* **242**(2), 238-242

178. Hubbard, S. C., and Brody, S. (1975) *Journal of Biological Chemistry* **250**(18), 7173-7181

179. Scarborough, G. A., and Nyc, J. F. (1967) *Biochimica et Biophysica Acta* **146**(1), 111-119

180. Garcia Gil, M., Alemany, S., Marin Cao, D., Castano, J. G., and Mato, J. M. (1980) *Biochemical and Biophysical Research Communications* **94**(4), 1325-1330

181. van Waarde, A., and van Hoof, P. J. M. (1985) *Biochimica et Biophysica Acta* **836,** 27-38

182. Smith, J. D. (1986) *Archives of Biochemistry & Biophysics* **246**(1), 347-354

183. Smith, J. D., and Law, J. H. (1970) *Biochimica et Biophysica Acta* **202**(1), 141-152

184. Smith, J. D. (1986) *Biochimica et Biophysica Acta* **878**(3), 450-453

185. Smith, J. D., and Giegel, D. A. (1982) *Archives of Biochemistry and Biophysics* **213**(2), 595-601

186. Moore, T. S. (1982) *Annual Review of plant Physiology* **33,** 235-259

187. Hanson, A. D., and Rhodes, D. (1983) *Plant physiology* **71,** 692-700

188. Mudd, S. H., and Datko, A. H. (1986) *Plant Physiology* **82,** 126-135

189. Datko, A. H., and Mudd, S. H. (1988) *Plant physiology* **88,** 854-861

190. Rolph, C. E., and Goad, L. J. (1991) *Physiologia Plantarum* **83,** 605-610

191. Hitz, W. D., Rhodes, D., and Hanson, A. D. (1981) *Plant physiology* **68,** 814-822

192. Williams, M., and Harwood, J. L. (1994) *Biochemical Journal* **304**(Pt 2), 463-468

193. Mudd, S. H., and Datko, A. H. (1987) *Plant physiology* **83,** S113

194. Schmid, K. M., and Ohlrogge, J. B. (1996) in *Biochemistry of Lipids, Lipoproteins and Membranes* (Vance, D. E., and Vance, J. E., eds), pp. 363-389, Elsevier, Amsterdam

195. Akesson, B. (1978) *FEBS Letters* **92**(2), 177-180

196. Hoffman, D. R., Marion, D. W., Cornatzer, W. E., and Duerre, J. A. (1980) *Journal of Biological Chemistry* **255**(22), 10822-10827

197. Pritchard, P. H., Chiang, P. K., Cantoni, G. L., and Vance, D. E. (1982) *Journal of Biological Chemistry* **257**(11), 6362-6367

198. Chiang, P. K., Richards, H. H., and Cantoni, G. L. (1977) *Molecular Pharmacology* **13,** 939-947

199. Schanche, J.-K., Schanche, T., and Ueland, P. M. (1982) *Biochimica et Biophysica Acta* **721,** 399-407

200. Chiang, P. K., and Cantoni, G. L. (1979) *Biochemical Pharmacology* **28,** 1897-1902

201. Sugiyama, K., Akachi, T., and Yamakawa, A. (1995) *Journal of Nutrition* **125**(8), 2134-2144

202. Mato, J. M., Alvarez, L., Corrales, F. J., and Pajares, M. A. (1994) in *The Liver: Biology and Pathobiology* (Arias, I. M., Boyer, J. L., Fausto, N., Jakoby, J. M., Schachter, D. A., and Shafritz, D. A., eds), 3rd Ed., pp. 461-469, Raven Press Ltd., New York

203. Geelen, M. J. H., Groener, J. E. M., De Haas, C. G. M., and Van Golde, L. M. G. (1979) *FEBS Letters* **105**(1), 27-30

204. Pritchard, P. H., Pelech, S. L., and Vance, D. E. (1981) *Biochimica et Biophysica Acta* **666**(2), 301-306

205. Pelech, S. L., Pritchard, P. H., Sommerman, E. F., Percival-Smith, A., and Vance, D. E. (1984) *Canadian Journal of Biochemistry & Cell Biology* **62**(4), 196-202

206. Marin-Cao, D., Alvarez Chiva, V., and Mato, J. M. (1983) *Biochemical Journal* **216**, 675-680

207. Castano, J. G., Alemany, S., Nieto, A., and Mato, J. M. (1980) *Journal of Biological Chemistry* **255**(19), 9041-9043

208. Mato, J. M., Alemany, S., Gil, M. G., Marin-Cao, D., Varela, I., and Castano, J. G. (1982) in *Biochemistry of S-Adenosylmethionine and Related Compounds* (Usdin, F., Borchardt, R. T., and Creveling, C. R., eds), pp. 187-200, Humana Press, Clifton, NJ

209. Pelech, S. L., Ozen, N., Audubert, F., and Vance, D. E. (1986) *Biochemistry & Cell Biology* **64**(6), 565-574

210. Ridgway, N. D., and Vance, D. E. (1989) *Biochimica et Biophysica Acta* **1004**(2), 261-270

211. Alemany, S., Varela, I., and Mato, J. M. (1981) *FEBS Letters* **135**(1), 11-14

212. Alemany, S., Varela, I., Harper, J. F., and Mato, J. M. (1982) *Journal of Biological Chemistry* **257**(16), 9249-9251

213. Merida, I., and Mato, J. M. (1987) *Biochimica et Biophysica Acta* **928**, 92-97

214. Pelech, S. L., Power, E., and Vance, D. E. (1983) *Canadian Journal of Biochemistry & Cell Biology* **61**(11), 1147-1152

215. Cui, Z., Shen, Y. J., and Vance, D. E. (1997) *Biochimica et Biophysica Acta* **1346**(1), 10-16

216. Cornatzer, W. E., Hoffman, D. R., and Haning, J. A. (1984) *Lipids* **19**(1), 1-4

217. Zaret, K. S. (1994) in *The Liver: Biology and Pathobiology* (Arias, I. M., Boyer, J. L., Fausto, N., Jakoby, W. B., Schachter, D. A., and Shafritz, D. A., eds), 3rd Ed., pp. 53-68, Raven Press Ltd., New York

218. Audubert, F., Pelech, S. L., and Vance, D. E. (1984) *Biochimica et Biophysica Acta* **792**(3), 348-357

219. Smith, J. D., and Ledoux, D. N. (1990) *Biochimica et Biophysica Acta* **1047**, 290-293

220. Pietrangelo, A., and Shafritz, D. A. (1994) in *The Liver: Biology and Pathobiology* (Arias, I. M., Boyer, J. L., Fausto, N., Jakoby, W. B., Schachter, D. A., and Shafritz, D. A., eds), 3rd Ed., pp. 85-98, Raven Press Ltd., New York

221. Houweling, M., Cui, Z., Tessitore, L., and Vance, D. E. (1997) *Biochimica et Biophysica Acta* **1346**(1), 1-9

222. Friedman, J. M., Chung, E. Y., and Darnell, J. E. J. (1984) *Journal of Molecular Biology* **179**, 37-53

223. Tessitore, L., Cui, Z., and Vance, D. E. (1997) *Biochemical Journal* **322**(Pt 1), 151-154

224. Weinhold, P. A., and Villee, C. A. (1965) *Biochimica et Biophysica Acta* **106**(3), 540-550

225. Weinhold, P. A. (1969) *Biochimica et Biophysica Acta* **187**(1), 85-93

226. Weinhold, P. A., and Sanders, R. D. (1971) *Biochemistry* **10**(6), 1090-1096

227. Weinhold, P. A., Skinner, R. S., and Sanders, R. D. (1973) *Biochimica et Biophysica Acta* **326**(1), 43-51

228. Pelech, S. L., Pritchard, P. H., and Vance, D. E. (1982) *Biochimica et Biophysica Acta* **713**(2), 260-269

229. Pelech, S. L., Pritchard, P. H., Sommerman, E. F., Percival-Smith, A., and Vance, D. E. (1983) *Canadian Journal of Biochemistry and Cell Biology* **62**, 196-202

230. Pelech, S. L., Pritchard, P. H., and Vance, D. E. (1981) *Journal of Biological Chemistry* **256**(16), 8283-8286

231. Nishimaki-Mogami, T., Suzuki, K., Okochi, E., and Takahashi, A. (1996) *Biochimica et Biophysica Acta* **1304**(1), 11-20

232. Ridgway, N. D., Yao, Z., and Vance, D. E. (1989) *Journal of Biological Chemistry* **264**(2), 1203-1207

233. Cui, Z., and Vance, D. E. (1996) *Journal of Biological Chemistry* **271**(5), 2839-2843

234. Houweling, M., Cui, Z., and Vance, D. E. (1995) *Journal of Biological Chemistry* **270**(27), 16277-16282

235. Andriamampandry, C., Freysz, L., Kanfer, J. N., Dreyfus, H., and Massarelli, R. (1991) *Journal of Neurochemistry* **56**(6), 1845-1850

236. Andriamampandry, C., Freysz, L., Kanfer, J. N., Dreyfus, H., and Massarelli, R. (1989) *Biochemical Journal* **264**(2), 555-562

237. Andriamampandry, C., Massarelli, R., and Kanfer, J. N. (1992) *Biochemical Journal* **288**, 267-272

238. Vance, J. E., and Vance, D. E. (1985) *Canadian Journal of Biochemistry & Cell Biology* **63**(8), 870-881

239. Yao, Z. M., and Vance, D. E. (1988) *Journal of Biological Chemistry* **263**(6), 2998-3004

240. Vance, J. E., Nguyen, T. M., and Vance, D. E. (1986) *Biochimica et Biophysica Acta* **875**(3), 501-509

241. Nishimaki-Mogami, T., Suzuki, K., and Takahashi, A. (1996) *Biochimica et Biophysica Acta* **1304**(1), 21-31

242. Vance, J. E., and Vance, D. E. (1986) *FEBS Letters* **204**(2), 243-246

243. Vance, J. E. (1991) *Journal of Lipid Research* **32**(12), 1971-1982

244. Verkade, H., Vonk, R. J., and Kuipers, F. (1995) *Hepatology* **21**, 1174-1189

245. Balint, J. A., Beeler, D. A., Kyriakides, E. C., and Treble, D. H. (1971) *Journal of Laboratory and Clinical Medicine* **77**, 122-133

246. Gregory, D. H., Vlahcevic, Z. R., Schatzki, P., and Swell, L. (1975) *Journal of Clinical Investigation* **55**, 105-114

247. Cronholm, T., Curstedt, T., and Sjovall, J. (1983) *Biochimica et Biophysica Acta* **753**, 276-279

248. Robins, S. J., and Brunengraber, H. (1982) *Jounal of Lipid Research* **23**, 604-608

249. Glenn, J. L., and Austin, W. (1971) *Biochimica et Biophysica Acta* **231**, 153-160

250. Fex, G. (1971) *Biochimica et Biophysica Acta* **231**, 161-169

251. Lyman, R. L., Hopkins, S. M., Sheehan, G., and Tinoco, J. (1969) *Biochimica et Biophysica Acta* **176**, 86-94

252. Salerno, D. M., and Beeler, D. A. (1973) *Biochimica et Biophysica Acta* **326**, 325-338

253. Sundler, R., and Akesson, B. (1975) *Biochemical Journal* **146**(2), 309-315

254. Samborski, R. W., Ridgway, N. D., and Vance, D. E. (1993) *Journal of Lipid Research* **34**(1), 125-137

255. Cui, Z., Houweling, M., and Vance, D. E. (1994) *Journal of Biological Chemistry* **269**(40), 24531-24533

256. Sastry, B. V., Statham, C. N., Axelrod, J., and Hirata, F. (1981) *Archives of Biochemistry & Biophysics* **211**(2), 762-773

257. Blusztajn, J. K., Zeisel, S. H., and Wurtman, R. J. (1985) *Biochemical Journal* **232**(2), 505-511

258. Crews, F. T., Hirata, F., and Axelrod, J. (1980) *Journal of Neurochemistry* **34**(6), 1491-1498

259. Hirata, F., Viveros, O. H., Diliberto, E. J., Jr., and Axelrod, J. (1978) *Proceedings of the National Academy of Sciences, USA* **75**(4), 1718-1721

260. Hirata, F., and Axelrod, J. (1978) *Proceedings of the National Academy of Sciences, USA* **75**(5), 2348-2352

261. Dudeja, P. K., Foster, E. S., and Brasitus, T. A. (1986) *Biochimica et Biophysica Acta* **875**(3), 493-500

262. Prasad, C., and Edwards, R. M. (1981) *Journal of Biological Chemistry* **256**(24), 1300-1303

263. Audubert, F., and Vance, D. E. (1983) *Journal of Biological Chemistry* **258**(17), 10695-10701

264. Higgins, J. A. (1981) *Biochimica et Biophysica Acta* **640**(1), 1-15

265. Higgins, J. A. (1980) *Biochemical Society Transactions* **8**(5), 648-649

266. Pajares, M. A., Alemany, S., Varela, I., Marin Cao, D., and Mato, J. M. (1984) *Biochemical Journal* **223**(1), 61-66

267. Pajares, M. A., Villalba, M., and Mato, J. M. (1986) *Biochemical Journal* **237**(3), 699-705

268. Villalba, M., Varetla, I., Merida, I., Pajares, M. A., Martinez del Pozo, A., and Mato, J. M. (1985) *Biochimica et Biophysica Acta* **847**, 273-279

269. Villalba, M., Pajares, M. A., Renart, M. F., and Mato, J. M. (1987) *Biochemical Journal* **241**, 911-916

270. Varela, I., Merida, I., Villalba, M., Vivanco, F., and Mato, J. M. (1985) *Biochemical and Biophysical Research Communications* **131**(1), 477-483

271. Tanaka, Y., Amano, F., Maeda, M., Nishijima, M., and Akamatsu, Y. (1990) *Japanese Journal of Medical Science & Biology* **43**(3), 59-73

272. Vance, D. E., and Ridgway, N. D. (1988) *Progress in Lipid Research* **27**(1), 61-79

273. Bansal, V. S., and Kanfer, J. N. (1985) *Biochemical & Biophysical Research Communications* **128**(1), 411-416

274. Hirata, F., and Axelrod, J. (1980) *Science* **209**(4461), 1082-1090

275. Hirata, F., Strittmatter, W. J., and Axelrod, J. (1979) *Proceedings of the National Academy of Science, USA* **76**(1), 368-372

276. Vance, D. E., and de Kruijff, B. (1980) *Nature* **288**(5788), 277-279

277. McKenzie, R. C., and Brophy, P. J. (1983) *FEBS Letters* **164**(2), 244-246

278. Moore, J. P., Johannsson, A., Hesketh, T. R., Smith, G. A., and Metcalfe, J. C. (1984) *Biochemistry Journal* **221**, 675-684

279. Vance, D. E., and Ridgway, N. D. (1986) in *Biological Methylation and Drug Design* (Borchardt, R. T., Creveling, C. R., and Ueland, P. M., eds), pp. 75-99, Humana Press, Clifton, NJ

280. Pike, M. C., and DeMeester, C. A. (1988) *Journal of Biological Chemistry* **263**(8), 3592-3599

281. Best, C. H., and Huntsman, M. E. (1932) *Journal of Physiology (London)* **75,** 405-412

282. Eagle, H. (1955) *Journal of Experimental Medicine* **102,** 595-600

283. Zeisel, S. H., and Blusztajn, J. K. (1994) *Annual Review of Nutrition* **14,** 269-296

284. Zeisel, S. H. (1981) *Annual Review of Nutrition* **1,** 95-121

285. Bussiere, M. (1997) Thesis in Department of Biochemistry, pp. 237, University of Alberta, Edmonton, AB

286. Bussiere, M., Campenot, R. B., Ure, D. R., Vance, J. E., and Vance, D. E. (1995) *Biochimica et Biophysica Acta* **1259**(2), 148-154

287. Weinhold, P. A., and Sanders, R. (1973) *Life Sciences* **13,** 621-629

288. Finkelstein, J. D., Martin, J. J., Harris, B. J., and Kyle, W. E. (1982) *Archives of Biochemistry and Biophysics* **218**(1), 169-173

289. Garcia-Perez, A., and Burg, M. B. (1991) *Journal of Membrane Biology* **119,** 1-13

290. Hoffman, D. R., Haning, J. A., and Cornatzer, W. E. (1981) *Canadian Journal of Biochemistry* **59**(7), 543-550

291. Tinoco, J., Endemann, G., Medwadowski, B., Miljanich, P., and Williams, M. A. (1979) *Lipids* **14**(12), 968-974

292. Kuksis, A., and Mookerjea, S. (1978) *Nutrition Reviews* **36**(7), 201-207

293. Blusztjan, J. K., and Zeisel, S. H. (1989) *FEBS Letters* **243,** 267-270

294. Rosenfeld, B. (1967) *Canadian Journal of Physiology and Pharmacology* **46,** 495-498

295. Blumenstein, J. (1964) *Canadian Journal of Biochemistry* **42,** 1183-1194

296. Haines, D. S. M. (1966) *Canadian Journal of Biochemistry* **44,** 45-57

297. Yao, Z. M., and Vance, D. E. (1990) *Biochemistry & Cell Biology* **68**(2), 552-558

298. Mookerjea, S., Park, C. E., and Kuksis, A. (1975) *Lipids* **10,** 374-382

299. Yao, Z. M., and Vance, D. E. (1989) *Journal of Biological Chemistry* **264**(19), 11373-11380

300. Griffith, W. H., and Wade, N. J. (1939) *Journal of Biological Chemistry* **131,** 567-577

301. Newberne, P. M., and Rogers, A. E. (1986) *Annual Review of Nutrition* **6,** 407-432

302. Haines, D. S. M. (1970) *Canadian Journal of Biochemistry* **48,** 885-892

303. Schneider, W. J., and Vance, D. E. (1978) *European Journal of Biochemistry* **85**(1), 181-187

304. Zeisel, S. H., Zola, T., DaCosta, K.-A., and Pomfret, E. A. (1989) *Biochemical Journal* **259,** 725-729

305. Pascale, R., Pirisi, L., Daino, L., Zanetti, S., Satta, A., Bartoli, E., and Feo, F. (1982) *FEBS Letters* **145**(2), 293-297

306. Yao, Z. M., Jamil, H., and Vance, D. E. (1990) *Journal of Biological Chemistry* **265**(8), 4326-4331

Chapter 2

Characterization of the Murine Phosphatidylethanolamine *N*-Methyltransferase-2 Gene

This chapter was published in the *Journal of Lipid Research*, **37**, 2341-2350 (1996).

2.1 Introduction

Phosphatidylcholine (PC) is the most abundant phospholipid in eukaryotic cells, functioning both as a structural component of membranes and as a source of second messengers. PC synthesis via CDP-choline occurs in all eukaryotic cells. An alternative pathway for PC synthesis, the conversion of phosphatidylethanolamine (PE) to PC, is largely confined to the liver (1). Three sequential methylation steps are catalyzed by phosphatidylethanolamine *N*-methyltransferase (PEMT), using *S*-adenosylmethionine as the methyl group donor. At least two PE methyltransferases exist in the liver: PEMT1, which is localized primarily to the endoplasmic reticulum, and PEMT2, which is present solely on the mitochondria-associated membrane (2). The reasons for hepatocytes having two distinct isoforms of PEMT are unclear. A full-length cDNA for PEMT2 has been isolated from rat liver (2).

Recent investigations in our laboratory suggest a strong inverse correlation between PEMT2 expression and liver cell growth. During embryogenesis, when hepatocytes grow and divide rapidly, PEMT activity is low (3). After birth, the rate of hepatocyte division slows (4), while PEMT activity rises considerably. This growth stage-specific regulation appears to occur at the level of gene expression (5). Rapidly dividing hepatoma-derived cell lines (McA-RH7777 and Hep G2) have almost undetectable PEMT activity. Expression of the rat liver PEMT2 greatly slows the growth rate of McA-RH7777 cells, but does not alter the growth of Chinese hamster ovary cells in culture (6). Chemically-induced liver tumours contain lower levels of PE methylation activity than normal liver, and no PEMT2 protein (Tessirote, L., Cui, Z. and Vance, D. E., unpublished observation). As well, PEMT2 expression is greatly decreased

during non-neoplastic liver growth (7). The growth inhibitory effects of PEMT2 appear to be directly related to the enzymatic conversion of PE to PC.

Using the rat PEMT2 cDNA as a probe, we have cloned and characterized the full-length mouse gene for PEMT2.

2.2 Materials and Methods

2.2.1 Isolation and Characterization of Genomic Clones

A 129/Sv strain mouse liver genomic DNA library (gift of Dr. Jim Stone, University of Alberta) was screened with random primer-labeled rat PEMT2 cDNA (2). Two positive clones, named 3 and 11, were isolated from an initial screen of approximately 2 x 10^7 plaques, and characterized by restriction mapping, DNA blotting and partial sequencing. A P1 library (Genome Systems Inc.) containing inserts up to 110 kb, was screened using polymerase chain reaction (PCR) primer pairs based on sequences from clones 3 and 11. Three P1 clones were isolated. One of the P1 clones, 2614, spanned the entire PEMT2 gene, according to DNA blotting and partial sequencing.

Sequencing was performed by the DNA Core facility, University of Alberta, using an automated sequencer (Applied Biosystems Inc./ Perkin Elmer model 373A). The results were analyzed using the Intelligenetics Geneworks program. Exon sequences were determined from at least two different clones, and intron/exon boundaries defined by comparison to the rat PEMT2 cDNA sequence. DNA preparations, restriction digests and ligations were performed

by standard methods. P1 DNA was isolated using a Qiagen DNA preparation kit following the manufacturer's protocol.

2.2.2 DNA Blotting

After restriction digestions, DNA fragments were electrophoresed through 1.0% agarose, transferred to Hybond N+ (Amersham) nylon membranes following the manufacturer's instructions, and probed with the rat PEMT2 cDNA radiolabeled with ^{32}P by random priming. The membranes were washed to a final stringency of 0.1X SSC (15 mM NaCl, 1.5 mM Na citrate, pH 7.0), 0.1% SDS at 52°C, and exposed to X-ray film.

2.2.3 RNA Primer Extension Assay

Total RNA was isolated from 8-week-old male 129/J strain mouse livers essentially as described by Chomczynski and Sacchi (8). 1 pmol of a 30-base oligonucleotide primer, rev-1: 5'-CTGTGGGGTCCACGTAACCCAGCAGCCAGC-3' was labeled with [γ-^{32}P]ATP at its 5'end by T4 polynucleotide kinase (Life Technologies Inc.), then hybridized to 40 µg of total mouse liver RNA at 30°C for 16 h (9). Extension reactions were catalyzed using SuperScript reverse transcriptase (Life Technologies Inc.). The reaction products were separated by electrophoresis (5% polyacrylamide in 8M urea), and visualized by autoradiography. The rat PEMT2 cDNA was sequenced with rev-1 as a primer using Sequenase 2.0 (Amersham) and following the manufacturer's instructions, and electrophoresed

alongside the extension products as a size marker. By comparing the position of the extension product bands to the sequencing ladder alongside, the length of the extension products, and hence the transcription start sites, were determined.

2.2.4 Reverse transcriptase-PCR

Primer extension products were eluted from the polyacrylamide gel by incubation for 48 h at 37°C in 0.5 M ammonium acetate, 1 mM EDTA, pH 8.0., and precipitated with ethanol. The primer extension products were then used as templates for PCR. Blank spots on the gel were also eluted as negative controls. The PCR reactions were performed in 50 µL volumes containing 0.25 mM dNTPs and 1.5 mM $MgCl_2$, for 30 cycles of 95°C for 30 s, 54°C for 30 s, and 74°C for 30 s, plus a final extension step of 2 min. Primers were rev-2, 5'-CAGAACCAGAAGGAAATGG-3'; for-1, 5'-TGGTAGAACTGCTATGTGCCG-3'; for-2, 5'-TTCCCTAACTACAGAACATTGC-3'. The PCR products were analyzed on a 1% agarose gel.

2.2.5 PEMT2 mRNA and protein analysis

Poly A-enriched RNA was isolated from adult male rat livers using a QuickPrep Micro mRNA Purification Kit (Pharmacia Biotech), according to manufacturer's instructions. PEMT2 mRNA was determined by RNA blot analysis. Briefly, RNA samples were electrophoresed and blotted onto a nylon membrane according to standard techniques (9). The blots were probed with rat

PEMT2 cDNA labeled by random priming, then washed under stringent conditions prior to autoradiography.

Homogenates of rat and mouse adult male livers were analyzed for the presence of PEMT2 protein by SDS-PAGE immunoblotting, exactly as previously described (2).

2.2.6 Chromosome Mapping

Interspecific backcross progeny were generated by mating (C57BL/6JEi x *M. spretus*) F_1 females with *M. spretus* males as described (10). Genomic DNA was digested with a selection of restriction enzymes, and probed with a 1.9 kb *Pst1* restriction fragment of clone 11 containing exon 5 radiolabeled by random priming. A single 6.6 kb band was detected in C57BL/6JEi genomic DNA digested with *Bgl2*, while two bands of 3.2 and 4.0 kb were found in *M. spretus* genomic DNA digested with *Bgl2*. This restriction fragment length polymorphism (RFLP) was followed in 94 N_2 offspring, and the segregation distribution pattern determined. Comparison to previously mapped loci and calculations of map distances were performed by the BC Panel Map Service at the Jackson Laboratory.

2.3 Results

2.3.1 Cloning and characterization of the mouse PEMT2 gene

Full-length rat PEMT2 cDNA was used to screen a library of 129/Sv strain male mouse liver genomic DNA, packaged in the bacteriophage vector

λDASH2. Two 15 kb clones, designated 3 and 11, were isolated and subcloned into pBluescriptII- SK+. DNA blots revealed that clone 3 hybridized to the 5' end of the PEMT2 cDNA, while clone 11 hybridized to the middle portion of the cDNA. The recognition sites of several restriction enzymes were mapped on both clones, using standard single and double digestion techniques. Using this restriction map, the fragments containing exons were identified by probing a DNA blot with rat PEMT2 cDNA, and sequenced. Consequently the number, size and location of exons in the PEMT2 gene were determined. Clone 3 contained two exons corresponding to the 5' end of the PEMT2 cDNA as predicted (Fig. 2.1). Clone 11 contained 3 adjacent exons. Since neither clone contained the 3' end exons, despite spanning 30 kb in total, a commercial 129/J mouse genomic DNA library, packaged in P1 phage-derived plasmid, was screened by PCR, using primers specific for exons I and V. One clone, 2614, that spanned the full length of the gene was isolated . Two additional clones, 2615 and 2616, spanned the 3' end only (Fig. 2.1). The P1 clones were subcloned and analyzed in the same manner as for the λ clones.

It appears that the murine PEMT2 gene is at least 35 kb long, with seven exons. Each exon was sequenced from at least two different clones (Figs. 2.1 & 2.2). These exon sequences from mouse are 92.8 % identical to the rat PEMT2 cDNA sequence at the nucleotide level, and 93.5 % identical at the amino acid level. The intron/exon boundaries in the genomic clones were deduced by sequence alignment to the rat PEMT2 cDNA (Table 2.1). The sequences of these boundary regions, especially the conserved GT and AG dinucleotides, match the consensus for pre-mRNA splicing recognition sites (11).

Genomic DNA from a 129/J mouse and clone 2614 DNA was digested with four different restriction enzymes and probed with rat PEMT2 cDNA in a DNA blot (Fig. 2.3). Identical bands were detected in both the genomic and the cloned DNA, indicating that clone 2614 accurately reflects the PEMT2 gene structure. The lack of additional bands of equal intensity in the genomic blot also indicates that mouse PEMT2 exists as a single-copy gene without pseudogenes. In other words, there is no complete or partial duplication of the PEMT2 gene elsewhere in the mouse genome. Addititional faint bands visible in the genomic blot are most likely due to allelic variation at the PEMT2 locus in the genomic DNA from the 129/J mouse, or segments of genomic DNA weakly homologous to PEMT2.

2.3.2 Mapping of the transcription initiation sites

The transcription start site(s) of the PEMT2 gene were determined by primer extension analysis, using the 30-base oligonucleotide rev-1, complementary to exon 2 of mouse PEMT2 mRNA. The extended products were sized by comparison to a DNA sequencing ladder. The results suggested two transcription start sites: a major one 139 base pairs upstream of the first ATG codon, and a second minor start site 148 base pairs upstream (Fig. 2.4a). The primer extension products were eluted from the sequencing gel and used as templates for PCR amplifications. A primer 3' to the putative start sites (for-1), in conjunction with another PEMT2-specific primer (rev-2), was able to amplify each extension product [Figs. 2.4b(i) and 2.4c], confirming the extension products as being PEMT2 cDNAs rather than the product of non-specific hybridization of the original 30mer. However, a primer specific to the region

beyond the putative start sites (for-2) failed to amplify the extension products [Fig. 2.4b(ii)]. This result confirms the location of the PEMT2 gene transcription start sites. Both transcription start sites have the same 4 base motif, CCAG. In both cases, the first nucleotide of the transcribed RNA is A, a purine. There does not appear to be a consensus TATA box (TATAA) in the region 30 bp upstream (12,13). A putative mouse PEMT2 cDNA based on the exon sequencing and primer extension data is shown in figure 2. This construct predicts PEMT2 transcripts of approximately 900 base pairs, and a 199-amino acid PEMT2 protein with a molecular mass of 22.5 kD.

2.3.3 Gene product analysis

To confirm the gene structure presented above, RNA and immunoblot analyses were performed on mouse liver samples. Probing mouse total RNA with rat PEMT2 cDNA revealed one band, indicating a single transcript of approximately 900 bp, highly identical to the rat PEMT2 transcript (Fig. 2.5a). The size of this transcript matches that predicted above. The nine base pair difference in transcript length due to the two different transcription start sites is too small to be resolved into two distinct bands.

Mouse and rat liver homogenates were tested for the presence of the PEMT2 protein by immunoblotting with an antibody raised against the C-terminus of the rat PEMT2 protein. In both mouse and rat samples, bands were detected indicating a protein of approximately 22 kD, close to the PEMT2 molecular mass of 22.5 kD predicted above. These results closely match those predicted from the gene structure presented above.

2.3.4 Chromosomal localization of PEMT2

The mouse chromosomal location of the PEMT2 gene (designated as the *Pempt2* locus) was determined by the interspecific backcross mapping method (10). Female C57BL/6J strain mice were crossed to male *M. spretus* strain mice, producing heterozygous offspring. These two inbred strains of mice were chosen because each strain carries a different allele for thousands of genetic loci. One method for differentiating the alleles is through restriction fragment length polymorphisms, or RFLPs. When genomic DNA from the two strains is digested with a restriction enzyme and DNA blotted with a probe for a locus, a difference is seen in the banding pattern for the two strains. This difference reflects a change in the nucleotide sequence of the locus between the two strains. Despite this genetic divergence, female offspring of the cross are fertile.

The females of the F_1 generation were then backcrossed to males of the parental *M. spretus* strain, producing 94 N_2 offspring. Each offspring contained one set of *M. spretus* chromosomes from the father and one set of chromosomes from the heterozygous mother. The maternal chromosomes underwent recombination during meiosis, producing a "mosaic" of *M. spretus* and C57BL/6J segments. Therefore at each locus, an N_2 offspring was either homozygous, with two *M. spretus* alleles, or heterozygous, with one *M. spretus* allele and one C57BL/6J allele. The genomic DNA of 94 N_2 offspring was isolated. For thousands of different loci whose positions were already known, the pattern of heterozygosity versus homozygosity of alleles (the segregation pattern) was determined in the N_2 generation. Each different locus produces its own pattern, reflecting the recombination points in the maternal chromosomes. Therefore, two loci closely linked will have a low frequency of recombination

between them, producing nearly identical patterns of heterozygosity versus homozygosity.

To map the *Pempt2* locus, it was necessary to find a suitable RFLP. A selection of restriction enzymes was screened with genomic DNA from C57BL/6J and *M. spretus* mice in DNA blots probed with a 1.9 kb fragment of clone 11 containing exon 5. The restriction enzyme *Bgl2* produced a distinct RFLP: one 6.6 kb band in C57BL/6J DNA and two bands of 3.2 and 4.0 kb in *M. spretus* DNA (Fig. 2.6a). This RFLP was followed in the genomic DNA of the 94 N_2 progeny. The *Bgl2* RFLP pattern of the *Pempt2* locus matched exactly that of the marker *D11Bir9*. Significant linkage was also found with the marker *D11Mit4* and the *Csfgm* locus (for colony-stimulating factor, granulocyte-macrophage). These results place the *Pempt2* locus on mouse chromosome 11, approximately 31 centimorgans from the centromere (Fig. 2.6, b & c). The distances between *Pempt2* and nearby loci in centimorgans (+/- standard error), and the most likely gene order (proximal to distal), are: *Csfgm* - 3.2(+/- 1.8) - *Pempt2* and *D11Bir9* - 4.3(+/- 2.1) - *D11Mit4*. This region of mouse chromosome 11 is syntenic with human chromosomes 5q and 17p.

2.4 Discussion

For phospholipid biosynthesis, murine PEMT2 is the first full-length mammalian gene that was cloned and characterized. More recently, the genes for rat choline kinase (partial) and mouse CTP:phosphocholine cytidylyltransferase (CT), have been isolated and characterized (14-16). These results for the mouse PEMT2 gene will allow us to further investigate the role of phospholipid methylation in whole animals by gene disruption.

The PEMT2 gene contains seven exons in at least 35 kb, and exists as a single copy. The exons vary in size between 75 and 259 base pairs (Fig. 2.2). The introns range from 0.3 kb to greater than 10 kb (Table 2.1). The PEMT2 gene is more than 30-fold larger than the fully-processed mRNA, although such a size difference is not particularly unusual (17). It will be interesting to learn if other genes involved in phospholipid biosynthesis in mammals have a similar size. Already, the mouse CT gene has been found to span 26 kb (16).The mouse exons are >92 % identical to the rat PEMT2 cDNA, suggesting strong conservation across species.

PEMT2 gene expression is regulated in both a tissue-dependent and developmental manner. The cloning of the PEMT2 gene, and in particular its 5' end, forms a foundation for the analysis of *cis*-acting elements that regulate its expression. Sequence analysis does not reveal a consensus TATA box sequence approximately 30 bp upstream of the 5'-most transcription start site. The presence of a functional TATA box in such a position would suggest that PEMT2 is a transcriptionally regulated gene, rather than a constitutively-expressed "housekeeping" gene (18). However, multiple transcription start sites and the presence of the MED-1 consensus sequence (GCTCCC) 110 bp downstream of the first start site place PEMT2 into a recently described family of TATA-less RNA polymerase II promoters (Fig. 2.4c) (19).

In the liver, the first appearance of PEMT2 mRNA occurs perinatally (5). Several other liver-specific genes are expressed in a similar pattern, including tyrosine aminotransferase (20) and α_1-antitrypsin (21). Liver-specific transcription factors, including members of the HNF (Heptaocyte Nuclear

Factor) family and C/EBP (CCAAT/Enhancer Binding Protein), have been implicated in gene expression during development (22). Figure 2.7 shows potential transcription factor binding sites identified in the immediate 5′ upstream region of the mouse PEMT2 gene. Interestingly, there are putative binding sites for both HNF5 and C/EBP. The role of these and other potential *cis*-acting elements in PEMT2 gene expression will be a focus of further investigation.

Using backcross mapping, the mouse *Pempt2* locus was localized to chromosome 11, approximately 31 centimorgans distal to the centromere. In contrast, the CTP:phosphocholine cytidylyltransferase gene was localized to mouse chromosome 16 (14). The same markers on mouse chromosome 11 that surround the *Pempt2* locus have their human counterparts on either chromosome 5q or 17p. Both 5q and 17p have been characterized as mutational "hot spots", frequently deleted in primary liver cancer (23). Therefore, we shall localize the PEMT2 gene in human chromosomes to investigate the role of this enzyme in human hepatocarcinogenesis.

We compared our map of chromosome 11 to a composite map showing the chromosomal location of many uncloned mouse mutations, in the hopes of finding a pre-existing mouse line carrying a mutated PEMT2 gene. The region of chromosome 11 near the *Pempt2* locus does not contain any mouse mutations that suggest a defect in phospholipid metabolism or liver growth. Defective mitochondria-associated membrane and low levels of PEMT2 protein were detected in mice carrying the *mnd* (for Motor Neuron Degeneration) gene (24), a mouse model for neuronal ceroid lipofuscinosis, or Batten's disease, which is characterized by defective lipid storage in neurons (25,26). However,

the *mnd* gene maps to the proximal end of mouse chromosome 8 (27), not 11, strongly suggesting that a fault in the PEMT2 gene is not the primary cause of the *mnd* defect. Therefore, further investigation is required to determine the link between decreased PEMT2 protein expression and the *mnd* phenotype.

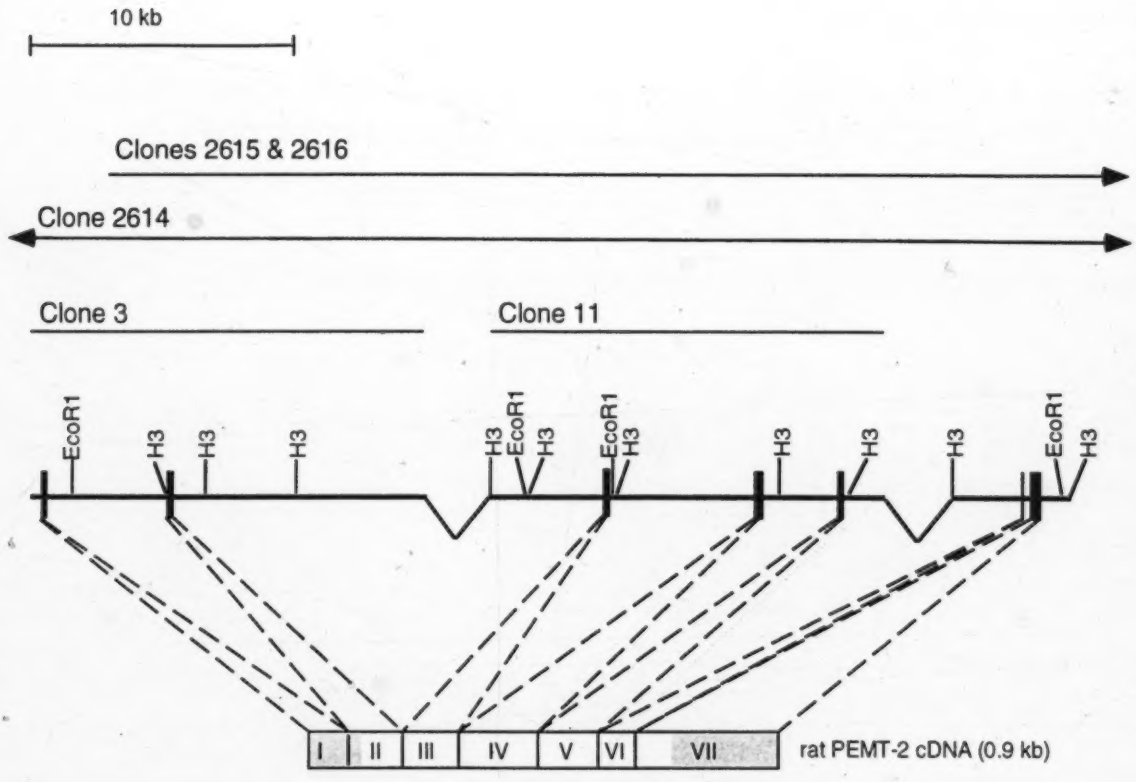


Figure 2.1 **The mouse PEMT2 gene**

Schematic representation of the genomic clones encompassing the mouse PEMT2 gene (*top*) and intron/exon organization of the gene (*bottom*). The position and size of the λ and P1 clones encompassing the 7 exons of the murine PEMT2 gene are shown as solid lines. Arrows indicate that the ends of the clones remain undetermined. Exons are represented by vertical solid boxes joined by solid lines representing introns. Divots in the introns indicate regions of unknown size. The relationship between the gene and the rat PEMT2 cDNA is shown below, depicting the relative sizes and organization of the exons. The shaded and unshaded areas represent untranslated and translated regions respectively.

Figure 2.2 **Putative mouse PEMT2 cDNA based on exon sequences.**

The sequences of all seven exons have been combined as in a fully spliced mouse PEMT2 mRNA. Junctions between exons are shown as vertical lines. The start of exon I is based on the primer extension results discussed in the text. Non-coding nucleotides are in lower case; coding nucleotides are in upper case. +1 indicates the first nucleotide of the translation start codon. Translation products of exons are shown below the nucleotide sequences. Nucleotide and amino acid changes from the rat PEMT2 cDNA sequence are underlined. The boldface type indicates the polyadenylation signal.

```
                          →exon I
-148                      aggtgcccagtgtggtagaactgctatgtgccgcct

-110 tcctttcagcttgtgggggttctgcagctgagttcatcatcagggtgacctgag

                                                  →exon II
-55  ctgtccctggaggctggctcccatttccttctggttctg|gctgatctcttcgtt

+1   ATGAGCTGGCTGCTGGGTTACATGGACCCCACAGAGCCCAGCTTTGTGGCGGCT
     M  S  W  L  L  G  Y  M  D  P  T  E  P  S  F  V  A  A

                                                  →exon III
 55  GTGATCACCATTGTGTTCAACCCACTCTTCTGGAATGTG|GTAGCGAGATGGGAG
     V  I  T  I  V  F  N  P  L  F  W  N  V   V  A  R  W  E

 109 CAGAGAACTCGGAAGCTGAGCAGAGCCTTCGGCTCCCCCCACCTAGCCTGCTAT
     Q  R  T  R  K  L  S  R  A  F  G  S  P  H  L  A  C  Y

                                                        →exon IV
 163 TCCCTGGGCATCTGCATCCTGCTTTTGAACATCCTCCGCTCCCACTG|CTTCACA
     S  L  G  I  C  I  L  L  L  N  I  L  R  S  H  C   F  T

 217 CAGGCTATGATGAGCCAGCCCAAGATGGAGGGCCTGGACAACCACACCACCTAC
     Q  A  M  M  S  Q  P  K  M  E  G  L  D  N  H  T  T  Y

 271 TTCCTGGGCCTTGCATTCCTAGGTTGGGGATTCGTGTTTGTGCTGTCCAGCTTC
     F  L  G  L  A  F  L  G  W  G  F  V  F  V  L  S  S  F

                                              →exon V
 325 TATGCACTGGGGTTCACTGGGACCTTTCTAG|GTGACTACTTTGGGATCCTCAAG
     Y  A  L  G  F  T  G  T  F  L  G   D  Y  F  G  I  L  K

 379 GAGTCCAGAGTGACCACATTTCCCTTCAGCGTGCTGGATAACCCCATGTACTGG
     E  S  R  V  T  T  F  P  F  S  V  L  D  N  P  M  Y  W

                                                  →exon VI
 433 GGCAGTACAGCCAACTACCTAGGCTGGGCACTCAT|GCATGCTAGTCCCACAGGC
     G  S  T  A  N  Y  L  G  W  A  L  M   H  A  S  P  T  G

 487 CTCCTGTTGACGGTGGTGGTGGCAATTGTCAACGTGGTTGCTCTCCTATATGAA
     L  L  L  T  V  V  V  A  I  V  N  V  V  A  L  L  Y  E

        →exon VII
 541 GA|GCCCTTCACTGCTGAGATCTACCGACAGAAAGCTACCAGGTTGCACAAAAGA
     E   P  F  T  A  E  I  Y  R  Q  K  A  T  R  L  H  K  R

 595 AGCTGAcagggccatgaaggatctttggaaagctggactggcctctcggctgcc
     S

 649 ccaagcaacaacccttccccagggagagcagcactggccactgtacctgtgcctt

 703 agaaaccagtcatgggggcctcaggcattatgctgtgtgactgctgagaccccc

 757 atccccaccatgtcccgacacactaataaaggcattgtgacctca
```

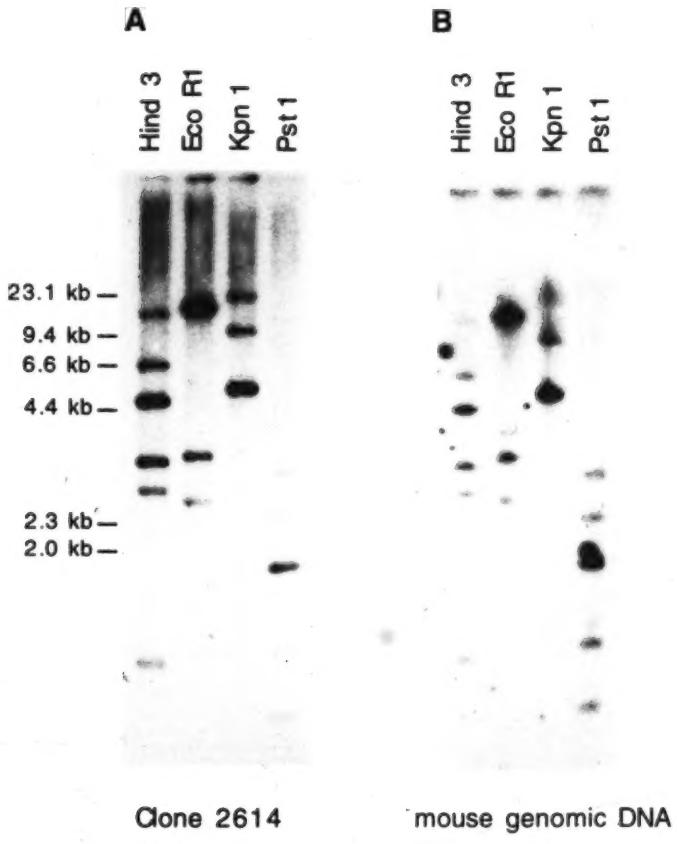


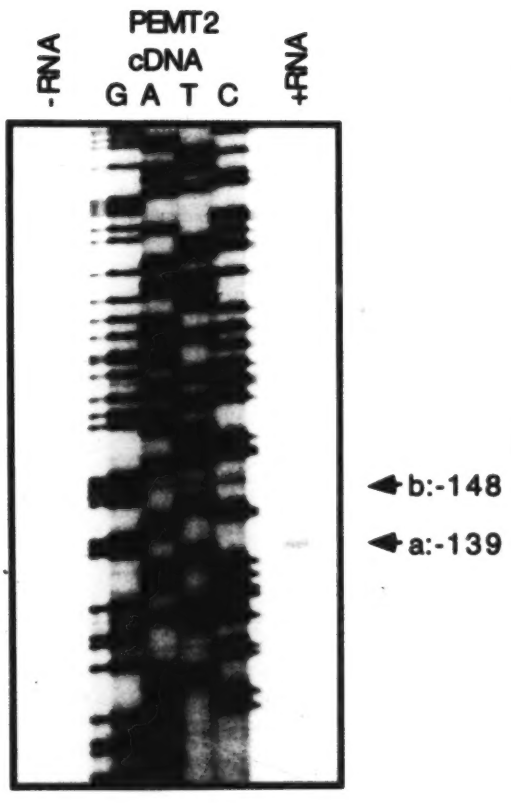
Figure 2.3 **DNA blotting of clone 2614 and mouse genomic DNA probed with rat PEMT2 cDNA.**

DNA from clone 2614 and 129/J strain mouse genomic DNA were digested with the restriction enzymes indicated at the top of the Figure, electrophoresed through a 1% agarose gel, alkali blotted and probed with rat PEMT2 cDNA. The numbers on the left side indicate the positions of DNA size markers.

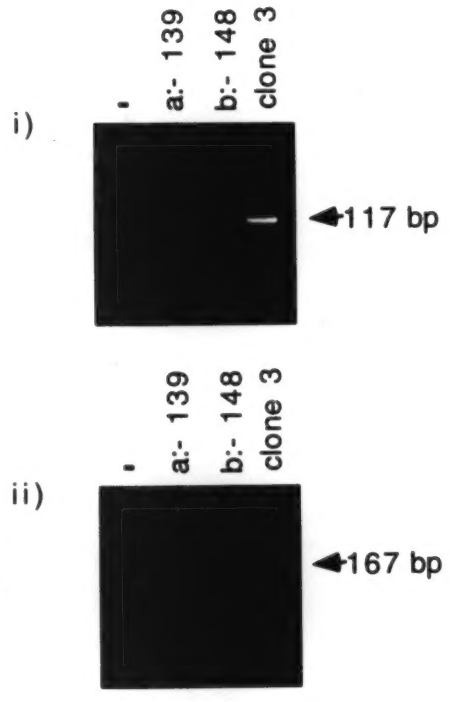
Figure 2.4 **Primer extension and reverse transcriptase-PCR results indicate two close transcription start sites.**

(A) RNA primer extension analyses were performed with and without mouse liver RNA (negative control) and rev-1, a 5'-labeled 30mer oligonucleotide based on the complement of the putative mouse mRNA sequence. The sizes of the extension products were determined by comparison to a DNA sequencing ladder of rat PEMT2 cDNA using the same oligonucleotide primer. Nucleotide numbers of each transcription start site relative to the first ATG codon are indicated on the right. (B) PCR was performed on the extension products using the primers rev-2/for-1(i) and rev-2/for-2 (ii). A region of the sequencing gel containing no extension products was used as a negative control. Clone 3 DNA was used as a positive control for the PCR reaction. (C) Sequence of the 5' region of the PEMT2 gene. The transcription start sites are denoted by asterices. The TATA box region and the MED-1 sequence are underlined. The positions of the primers used in the PCR reactions are shown as arrows. The numbers refer to the positions of the various nucleotides relative to the first ATG codon.

A



B



C

for-2 >

-184:ttcc<u>ctaacta</u>cagaacattgctctgaagtgtgtccaggtgcccagtgtg

TATA?
(-179 to -174)

(-148) (-139)

for-1 >

tggtagaactgctatgtgccgccttcctttcagcttgtgggggttctgcagctgagtt

< rev-2

catcatcagggtgacctgagctgtccctggaggc<u>tggctc</u>ccatttccttctggttc:-18

MED-1
(-38 to -33)

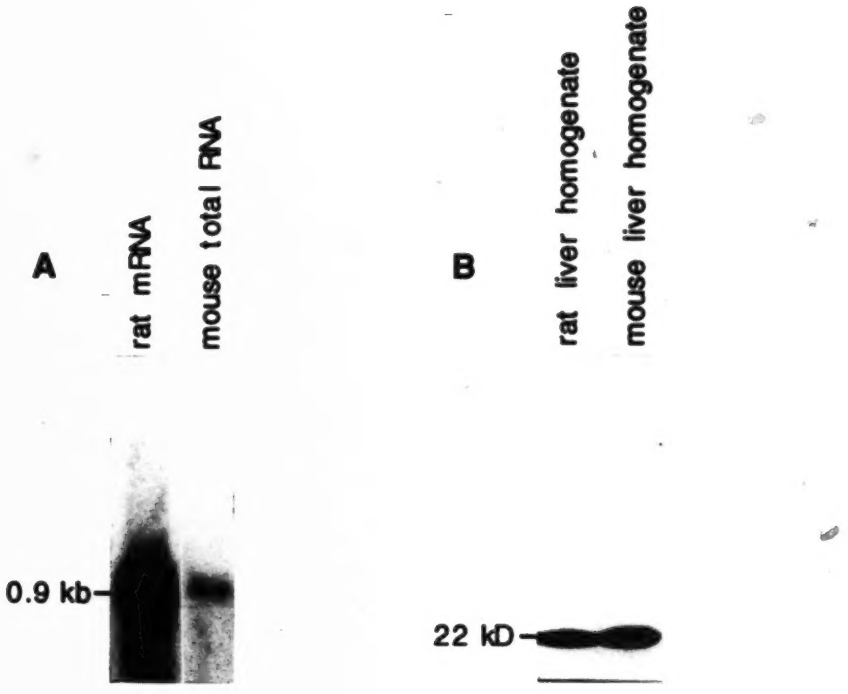


Figure 2.5 **The PEMT2 gene product**

(A) RNA blot of mouse liver total RNA and rat liver mRNA. RNA samples were isolated, electrophoresed, blotted, and probed with rat PEMT2 cDNA. The size of the bands is indicated on the left. (B) SDS-polyacrylamide gel electrophoresis immunoblot of mouse and rat liver homogenates. Antibody raised against the C-terminus of rat PEMT2 detects 22 kD proteins from both rat and mouse proteins.

Figure 2.6 ***Pempt2* maps to mouse chromosome 11**

(A) DNA blot of genomic DNA from C57BL/6JEi, *M. spretus* and an F_1 offspring of the two parental strains, using an exon 5-containing probe, reveals a polymorphism in the *Pempt2* locus. (B) The segregation patterns of the *Pempt2* polymorphism and flanking markers in 94 backcross progeny were determined. Each column represents chromosome 11 inherited from the (C57BL/6JEi x *M. spretus*) F_1 parent. Black boxes represent the C57BL/6JEi allele, and the white boxes represent the *M. spretus* allele. The number of offspring inheriting each type of chromosome is indicated at the bottom of each column. (C) The figure on the left is a partial map of mouse chromosome 11, showing the position of the *Pempt2* locus in relation to the nearby markers with which it was mapped. Recombination distances between the markers are shown in centimorgans on the left. The figure on the right is a partial composite linkage map of mouse chromosome 11 (data from GBASE, 1995, The Jackson Laboratory, Bar Harbor, ME). The numbers on the left indicate the distance of each locus from the centromere, in centimorgans. The positions of these same loci on human chromosomes are shown on the right.

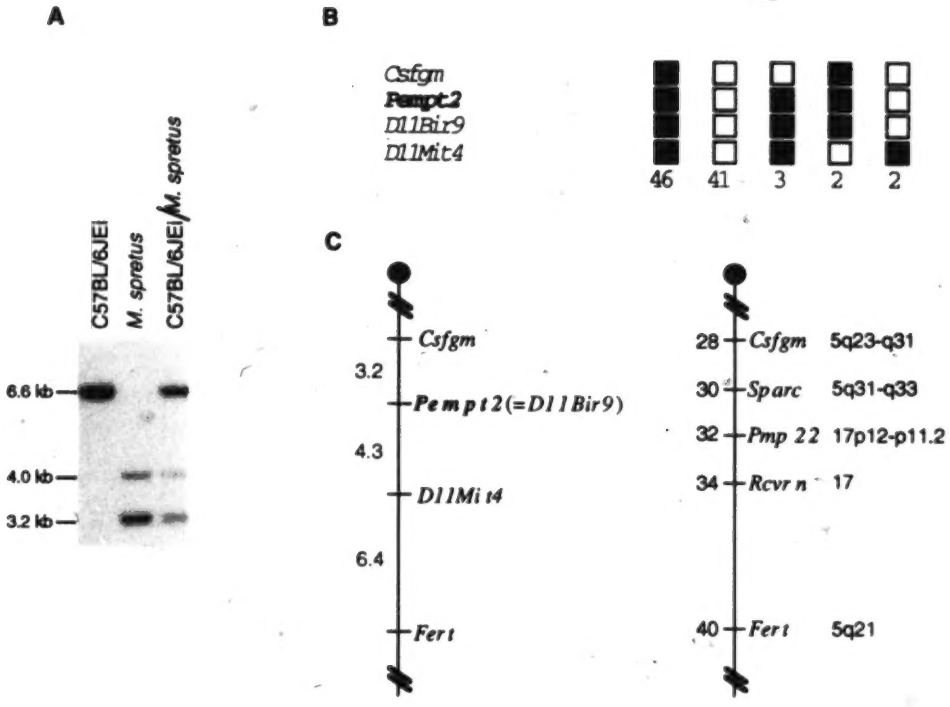

A
C57BL/6JEI
M. spretus
C57BL/6JEI/M. spretus
6.6 kb
4.0 kb
3.2 kb
B
Csfgm
Pempt2
D11Bir9
D11Mit4
46
41
3
2
2
C
Csfgm
3.2
Pempt2(=D11Bir9)
4.3
D11Mit4
6.4
Fert
28 Csfgm 5q23-q31
30 Sparc 5q31-q33
32 Pmp 22 17p12-p11.2
34 Rcvrn 17
40 Fert 5q21

Figure 2.7 **Sequence analysis of the murine PEMT2 exon I and its 5' flanking region**

Candidate binding sites for transcription factors were identified using the *Findpatterns* program of the GCG sequence analysis software package (Genetics Computer Group Inc., Madison, WI), with a combined database from TFD (release 7.5, 3/96) and Transfac (release 2.5, 1/96). AP-3: binding site for AP-3; C/EBP: binding site for the CCAAT/enhancer binding protein; E2A: E box binding site for E2A; GR: glucocorticoid receptor binding element; H-APF-1: interleukin-6 responding element; HNF5: binding site for Hepatic Nuclear Factor 5; MyoD: binding site for MyoD; PEA3: primary target of signal transduction responding to TPA, EGF and serum; PPAR: binding site for Peroxisome Proliferator Activated Receptor; T3: binding site for the Thyroid Hormone Receptor.

```
1    agggagggcacagtgtatgaaatggggcagaaaggatgccttaatagtaa
           GR

51   acatggtgcctccccctcttgggagtgtagacctaggtagagaggttcca

                                                  C/EBP
101  gtaccttgcttctttctttcaggccacagggctcttgtgggaattgcccag
                                                 AP-3

151  cgaatgttcctggatctgctggtaattcctggacactgggatagctgggga
                                                    H-APF-1

                              C/EBP
201  agagggcgaagagtatgcaattgcagagggccactgtatgggatgaagga
                         C/EBP

251  acctgtccccggtctgagacagccccactctgtcccttttatcctttgat
       GR                                GR

301  gtgtatggtacatgtcacagagaggcccatgactttcaggtgatcaatgc
           GR                                MyoD

351  aaccctggctgtctggcactctggctgttttgttggatgtcacaatgcag
                                  HNF5

401  cttgcaccctgggtgctgttgacccccctgcaaatcattccctaactacag

                                  |→Exon 1
451  aacattgctctgaagtgtgtccCAGGTGCCCAGTGTGGTAGAACTGCTATG
      GR                  GR    MyoD

       Sp1
501  TGCCGCCTTCCTTTCAGCTTGTGGGGGTTCTGCAGCTGAGTTCATCATCA
   C/EBP   PEA3                          E2A

551  GGGTGACCTGAGCTGTCCCTGGAGGCTGGCTCCCATTTCCTTCTGGTTCT
       PPAR/T3     GR                       PEA3

601  Ggt...intron 1...
```

Intron	5' splice donor	Intron length (kb)	3' splice acceptor	Codon phase	Amino acid at splice site
1	**GTTCTGGTAGGT**	5.0	TTGCAG**GCTGAT**	–	–
2	**AATGTGGTGAGT**	>13.0	ATGCAG**GTAGCG**	0	32 - Val/Val
3	**CCACTGGTAAGC**	5.7	CCACAG**CTTCAC**	I	70 - Cys
4	**TTCTAGGTAAGA**	2.9	CCACAG**GTGACT**	I	119 - Gly
5	**ACTCATGTGAGT**	>4.4	CTGCAG**GCATGC**	I	156 - Met
6	**TGAAGAGTGAGT**	0.3	CCACAG**GCCCTT**	I	181 - Glu

Table 2.1 **Exon/intron organization of the mouse PEMT2 gene**

Nucleotide sequences were determined by sequencing exon containing subclones of the λ and P1 genomic clones (see Methods). Exon sequences are boldface. The invariant GT (5') and AG (3') dinucleotides at the ends of the introns are underlined. The position of each splice site relative to a codon is shown as the codon phase. 0 indicates a splice site between codons, I indicates a splice site after the first nucleotide of a codon, and II indicates a splice site after the second nucleotide of a codon. Amino acids are numbered from the initiator methionine.

References

1. Bremer, J., and Greenberg, D. M. (1961) *Biochimica et Biophysisca Acta* **46,** 205-216

2. Cui, Z., Vance, J. E., Chen, M. H., Voelker, D. R., and Vance, D. E. (1993) *Journal of Biological Chemistry* **268**(22), 16655-16663

3. Pelech, S. L., Power, E., and Vance, D. E. (1983) *Canadian Journal of Biochemistry & Cell Biology* **61**(11), 1147-1152

4. Grisham, J. W. (1962) *Cancer Research* **22,** 842-849

5. Cui, Z., Shen, Y. J., and Vance, D. E. (1997) *Biochimica et Biophysica Acta* **1346**(1), 10-16

6. Cui, Z., Houweling, M., and Vance, D. E. (1994) *Journal of Biological Chemistry* **269**(40), 24531-24533

7. Tessitore, L., Cui, Z., and Vance, D. E. (1997) *Biochemical Journal* **322**(Pt 1), 151-154

8. Chomcynski, P., and Sacchi, N. (1987) *Analytical Biochemistry* **162,** 156-159

9. Kingston, R. E. (1987) in *Current Protocols in Molecular Biology*, pp. 4.1.1 -4.10.8, John Wiley & Sons, New York

10. Rowe, L. B., Nadeau, J. H., Turner, R., Frnakel, W. N., Letts, V. A., Eppig, J. T., Ko, M. S., Thurston, S. J., and Birkenmeier, E. H. (1994) *Mammalian Genome* **5,** 253-274

11. Mount, S. M. (1982) *Nucleic Acids Research* **10,** 459-472

12. Simon, M. C., Fisch, T. M., Benecke, B. J., Nevins, J. R., and Heintz, N. (1988) *Cell* **52,** 723-729

13. Wefald, W. E., Devlin, B. H., and Williams, R. S. (1990) *Nature* **344,** 260-262

14. Rutherford, M. S., Rock, C. O., Jenkins, N. A., Gilbert, D. J., Tessner, T. G., Copeland, N. G., and Jackowski, S. (1993) *Genomics* **18**(3), 698-701

15. Uchida, T. (1994) *Journal of Biochemistry* **116**(3), 508-518

16. Tang, W., Keesler, G. A., and Tabas, I. (1997) *Journal of Biological Chemistry* **272**(20), 13146-13151

17. Watson, J. D., Hopkins, N. H., Roberts, J. W., Steitz, J. A., and Weiner, A. M. (1987) *Molecular Biology of the Gene*, Benjamin/Cummings, Menlo Park, CA

18. Kageyama, R., Merlino, G. T., and Pastan, I. (1989) *Journal of Biological Chemistry* **264,** 15508-15514

19. Ince, T. A., and Scotto, K. W. (1995) *Journal of Biological Chemistry* **270,** 30249-30252

20. Nitsch, D., Boshart, M., and Schultz, G. (1993) *Procéedings of the National Academy of Science, USA* **90,** 5479-5483

21. Courtois, G., Morgan, J. G., Campbell, L. A., Fourel, G., and Crabtree, G. R. (1987) *Science* **238,** 688-692

22. Xanthopoulos, K. G., and Mirkovitch, J. (1993) *European Journal of Biochemistry* **216,** 353-360

23. Ozturk, M. (1995) in *Molecular Biology and Biochemistry*, pp. 516-518, VCH, New York

24. Vance, J. E., Stone, S. J., and Faust, J. R. (1997) *Biochimica et Biophysica Acta* **1344**(3), 286-299

25. Bronson, R. T., Lake, B. D., Cook, S., Taylor, S., and Davisson, M. T. (1993) *Annals of Neurology* **33,** 381-385

26. Chang, B., Bronson, R. T., Hawes, N. L., Roderick, T. H., Peng, C., Hageman, G. S., and Heckenlively, J. R. (1994) *Investigations in Opthamology and Vision Science* **35,** 1071-1076

27. Messer, A., Plummer, J., Maskin, P., Coffin, J. M., and Frankel, W. M. (1992) *Genomics* **13,** 797-802

Chapter 3

Disruption of the Murine Gene Encoding Phosphatidylethanolamine *N*-Methyltransferase

This chapter was published in the *Proceedings of the National Academy of Science, USA*, **94**, 12880 - 12885 (1997).

3.1 Introduction

Phosphatidylcholine (PC), the most abundant mammalian phospholipid, is synthesized in all nucleated cells via a three step process: phosphorylation of intracellular choline, conversion of phosphocholine to CDP-choline, which reacts with diacylglycerol to form PC (1). A second pathway for the biosynthesis of PC is the methylation of phosphatidylethanolamine (PE). This pathway, first elucidated in 1961, occurs in mammals to a significant degree only in the liver (2,3). *S*-adenosylmethionine (AdoMet) serves as the methyl group donor for the three reactions that convert the ethanolamine head group of PE to choline via two lipid intermediates: phosphatidylmonomethylethanolamine (PMME) and phosphatidyldimethylethanolamine (PDME). Unlike yeast, which use two distinct enzymes to convert PE to PC, only one enzyme in mammals, phosphatidylethanolamine *N*-methyltransferase (PEMT), is necessary for the complete conversion of PE to PC (4-6).

PEMT was first purified from rat liver in 1987, and shown to be a single enzyme of approximately 19 kDa (5). Using oligonucleotides based on the N-terminal amino acid sequence of the purified protein, a cDNA encoding PEMT was cloned and expressed (6). Interestingly, the cDNA encoded a protein which, when detected by an anti-C-terminal peptide antibody, differed in its subcellular localization from that of the majority of PEMT activity. This result led to the hypothesis that two isoforms of PEMT exist: PEMT1, localized to the endoplasmic reticulum and generating the majority of PEMT activity, and PEMT2, corresponding to the cloned cDNA, which resides on mitochondria-associated membranes (6,7). Both enzymes are capable of catalyzing all three methylation

reactions. The difference between these two isoforms is currently being investigated.

The structure and chromosomal localization of the mouse PEMT2 gene has been determined (8). The gene consists of seven exons between 75 and 260 bp long, spread over at least 35 kb, with the translation start site encoded by the second exon. Interspecific backcross mapping placed the gene, named *Pempt2*, on mouse chromosome 11, approximately 31 centimorgans from the centromere.

The physiological role of PE methylation in the liver is unclear. The majority of PC in the liver is synthesized via the CDP-choline pathway (also commonly called the Kennedy pathway), with PEMT generating 20 to 40% of liver PC (9). One obvious function for PEMT would be the endogenous generation of choline, which could be used for PC biosynthesis via the CDP-choline pathway, for the biosynthesis of the neurotransmitter acetylcholine, and as a source of betaine, which supplies methyl groups to the 1-carbon pool. As well, PEMT-derived PC may be specifically targeted for secretion, either with bile or with lipoproteins. Initial studies suggest that PEMT-derived PC is secreted with lipoproteins, but PEMT activity is not strictly required (10-12). However, inhibition of PE methylation with fibrates in primary rat hepatocytes alters lipidation of apoB48-containing lipoproteins (13). More recently, a role for PEMT in the regulation of liver growth has been suggested. When hepatocytes undergo non-neoplastic division after injection of lead nitrate, during embryonic development and following partial hepatectomy, PEMT expression is decreased (14-16).

Targeted disruption of gene(s) encoding PEMT in mice seemed a particularly suitable approach to providing fundamental insight into the function of PEMT, since PEMT is not expressed in the liver until birth (15), hence embryonic lethality would not be expected. As well, the liver retains the CDP-choline pathway, which we hypothesized could compensate for the loss of PEMT by generating sufficient PC for hepatocyte viability. Since PEMT is not detectable in other tissues, serious non-hepatic consequences would not be expected.

We now report the construction of mice in which *Pempt2* has been disrupted. No obvious pathophysiological phenotype developed in these mice up to 10 months old. Mice lacking *Pempt2* expression breed normally. Thus it is apparent that this gene is not required for normal growth and reproduction. This is the first report of the disruption of a phospholipid biosynthetic gene in any animal species.

3.2 Materials and Methods

3.2.1 Generation of Phosphatidylethanolamine N-Methyltransferase-Deficient Mice.

A sequence-replacement type gene targeting vector was constructed from a 6 kb *BamH1* subclone of λ clone 3 containing exon 2 of the mouse *Pempt2* gene. A cassette containing a neomycin-resistance gene (*neo*) driven by the *pgk* promoter was inserted at unique *Xho1* and *Bcl1* sites in the subclone, replacing the majority of exon 2, including the translation start site, and the extreme 5' end of intron 1 (Fig. 3.1). Therefore, the long arm of the vector consisted of the 3' end of exon 2 and the 5' end of intron 2. The short arm consisted of the remaining 3'

end of intron 1. A thymidine kinase (*tk*) gene was ligated to the 5' end of the targeting vector. The vector was electroporated into E14 embryonic stem (ES) cells (17). Homologous recombinants were identified by DNA blot analysis of *EcoR1*-cleaved genomic DNA, using a 1 kb *EcoR1-Xho1* fragment from intron 1, 5' to the targeting vector, as a probe. Identifications were confirmed by probing with a 800 bp *HindIII-Kpn1* fragment 3' to the targeting vector. One *Pempt2* gene-disrupted clone was used to generate PEMT2-deficient mice using standard techniques (18).

3.2.2 Tissue Collection

Mice were sacrificed following an overnight fast, and liver and other organs removed. Tissues were cut into small pieces with scissors, then homogenized with a glass-Teflon homogenizer in five volumes of 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1.0 mM phenylmethylsulfonyl fluoride, 1.0 mM EDTA, 2.0 mM dithiothreitol, 0.025% sodium azide. Plasma was isolated by centrifugation following blood collection by cardiac puncture, with EDTA as an anti-coagulant.

3.2.3 Determination of enzyme mass and activity

Total protein mass in homogenates was measured using the bicinchoninc acid - protein assay (Pierce), with bovine serum albumin as standard. To determine enzyme mass, homogenates were boiled for 5 min in Laemmli buffer (19), then separated electrophoretically on a 12% polyacrylamide gel containing 0.1% SDS (50 µg protein per lane). Proteins were transferred to Immobilon-P membranes (Millipore), and probed with an anti-PEMT2-specific antibody (6). Bands were

visualized using the ECL™ system following manufacturer's instructions (Amersham).

Homogenates were centrifuged at 600 x g for 5 min to remove unbroken cells, and the supernatant was assayed for PEMT activity (20). Methylated, chloroform-soluble products were separated by thin-layer chromatography on silica gel G60 plates with chloroform/propionic acid/n-propanol/water (30/20/60/10) as solvent. Bands were visualized by iodine vapor, and those corresponding to methylated PE products (PMME, PDME and PC) were scraped and radioactivity measured.

For CTP:phosphocholine cytidylyltransferase (CT) activity measurements, soluble and membrane fractions of the 600 x g supernatant were separated by centrifugation at 350,000 x g for 15 min. Fractions were assayed in the presence of PC/oleate vesicles, as previously described (21).

3.2.4 Phospholipid analyses

Phospholipids were isolated from total homogenates by the method of Bligh and Dyer (22), then separated by thin-layer chromatography with chloroform/methanol/acetic acid/water (50/30/8/4) as the solvent. Bands were visualized with iodine vapor, then scraped and phosphorous content measured (23).

3.2.5 In vivo *metabolism of [^{3}H]methionine*

62.5 µCi of [L-*methyl*-^{3}H]-labeled methionine was diluted to a final volume of 200 µL with phosphate-buffered saline, then injected into mice through the tail vein. After 5 min, 1 h and 24 h, mice were sacrificed, organs and plasma were harvested as described above, and choline-containing phospholipids (PC, lyso-PC and sphingomyelin) isolated by thin-layer chromatography. Phospholipids were analyzed for mass and radiolabel incorporation.

3.3 Results

3.3.1 Generation of Pempt2-deficient mice

A gene targeting vector was designed and constructed to interrupt exon 2 of the *Pempt2* gene, including the translation start site (Fig. 3.1A). The vector was electroporated into ES cells, and 7 targeted ES cell clones out of 131 neomycin-resistant clones were identified by DNA blot analysis. One of these clones was used to generate chimeric mice (strains 129/J and C57BL/6) capable of germline transmission of the altered *Pempt2* allele. The chimeric mice were bred with C57Bl/6 females to generate mice heterozygous for the disrupted *Pempt2* gene. These mice were interbred to generate all three genotypes: *Pempt2*(+/+) carrying two copies of the normal allele, *Pempt2*(+/-) carrying one normal and one disrupted allele, and *Pempt2*(-/-), carrying two copies of the disrupted allele (Fig. 3.1B). From 110 offspring, 32 *Pempt2*(+/+), 54 *Pempt2*(+/-) and 24 *Pempt2*(-/-) were identified. This result is close to the expected Mendelian ratio of 1:2:1, suggesting that disruption of *Pempt2* does not affect gamete viability.

3.3.2 Phosphatidylethanolamine N-methyltransferase 2 is absent from Pempt2 (-/-) mice

We investigated whether or not disruption of the *Pempt2* gene eliminated expression of PEMT2 protein. Immunoblot analysis was performed on liver homogenates from mice, using an antibody against the C-terminus of rat PEMT2 (6). The 19 kDa protein was present in *Pempt2*(+/+) and (+/-) mice, but completely absent from *Pempt2*(-/-) mice (Fig. 3.1C). The band intensity appeared reduced in heterozygotes compared to normal mice, suggesting a gene dosage effect.

PEMT activity was measured in liver homogenates from mice of all three genotypes. With PMME and PDME as substrates, PEMT activity in the homozygous knockout mice was less than 0.3% of that seen in normal mice (Fig. 3.2). Activity in heterozygotes was approximately half that of wild-type mice, suggesting a gene dosage effect on activity. This result demonstrates that both PEMT1 and PEMT2 are encoded by *Pempt2*. With PE as substrate, PEMT activity of the normal mice was ~10% of that against PMME and PDME. It has previously been reported that PE is a relatively poor substrate for PEMT compared to PMME and PDME (5). However, homozygous knockout mice retained ~20% of the PE methylation activity seen in their normal littermates. This residual activity appears to be quantitatively unimportant for conversion of PE to PC. PEMT activity in other tissues was less than 1% that found in the liver.

3.3.3 PC and PE levels in liver are minimally affected by elimination of Pempt2

Lipids were extracted from liver homogenates, separated by thin-layer chromatography, and quantitated. The PC content was not significantly altered, and PE levels were raised slightly, from 30.2% of total phospholipids in *Pempt2*(+/+) mouse livers, to 32.4% in *Pempt2*(+/-) mouse livers, to 33.85% in *Pempt2*(-/-) mouse livers (Fig. 3.3A). The level of total liver phospholipids did not change significantly according to genotype, varying from 83.8±1.8 nmol/mg protein in *Pempt2*(+/+) mice, to 82.7±4.2 nmol/mg protein *Pempt2*(+/-) mice to 86.3±3.9 nmol/mg protein in *Pempt2*(-/-) mice. As well, levels of other phospholipid species were unaffected.

3.3.4 CTP:phosphocholine cytidylyltransferase activity is increased in livers of Pempt2-disrupted mice

To determine whether the CDP-choline pathway for PC biosynthesis is stimulated to maintain hepatic PC levels in PEMT-deficient mice, the activity of CTP:phosphocholine cytidylyltransferase, which catalyzes the rate limiting step in this pathway (24-26), was measured. The enzyme exists in two forms: a soluble, inactive form and a membrane- bound, active form. In livers of *Pempt2*(-/-) mice, membranous CT activity was increased by 60% ($P<0.05$), from 1.45 nmol/min/mg protein in normal mice to 2.32 nmol/min/mg protein in homozygous knockouts (Fig. 3.4). Heterozygotes showed intermediate values. Thus, it appears that mice compensate for the loss of PEMT activity by stimulation of the CDP-choline pathway to maintain liver PC levels. No significant changes in CT activity were observed in extrahepatic tissues.

3.3.5 Incorporation of [^{3}H]methionine into choline-containing phospholipids

We investigated the extent to which the conversion of PE to PC *in vivo* was impaired in *Pempt2*(+/-) and (-/-) mice. Mice of each genotype were injected with [L-*methyl*-^{3}H]methionine via the tail vein. Organs and plasma were harvested either 5 min., 1 h or 24 h after injection, and radiolabeling of PC, lysoPC and sphingomyelin was measured. Labeled PC (90000 dpm/μmol choline-containing phospholipid) was recovered from the liver, but not plasma of the *Pempt2*(+/+) mouse 5 min. after injection. Less labeled PC (30929 dpm/μmol choline-containing phospholipid) was recovered from the liver of the *Pempt2*(+/-) mouse, and the *Pempt2*(-/-) mouse contained less than 1% (738 dpm/mmol choline-containing phospholipid) of label associated with PC in its liver compared to the *Pempt2*(+/+) mouse. After 1 h, the level of radiolabeled choline-containing phospholipids in liver was highest in the *Pempt2*(+/+) mouse (Fig. 5a), less in the *Pempt2*(+/-) mouse (62.0% of +/+), and much less in the *Pempt2*(-/-) mouse (4.4% of +/+) (Fig. 3.5A). This result correlates with the pattern of PEMT activity in liver homogenates (Fig. 3.2). In all genotypes, >85% of radioactivity was in PC, with minor amounts in lysoPC and sphingomyelin. After 1 h, the plasma also contained significant levels of radiolabeled choline-containing phospholipids, primarily PC (79.9%) and lysoPC (20.1%), suggesting that PC and lysoPC (with a choline moiety derived from PE methylation) are secreted from the liver into the bloodstream. The level of radiolabeled choline-containing phospholipids in other tissues was minimal, consistent with PEMT being a liver-specific enzyme.

Between 1 and 24 h after injection of [L-*methyl*-^{3}H]methionine, the level of labeled PC, lysoPC and sphingomyelin in the liver of the *Pempt2*(+/+) mouse decreased, falling slightly below that in the *Pempt2*(+/-) (Fig. 3.5B). Concurrently,

the level of radiolabeled choline-containing phospholipids in other organs examined increased significantly, in both normal and heterozygous mice. At all times, incorporation of label into brain phospholipids was minimal. This result suggests that choline derived from PE methylation in the liver is distributed throughout the body following secretion, where it is found in PC, lysoPC and sphingomyelin. At all times, the level of radiolabeled choline-containing phospholipids was low in all tissues of the *Pempt2*(-/-) mouse.

Our ability to trace the incorporation of [^{3}H]methyl groups into aqueous choline is compromised by the promiscuity of methionine as a precursor for other water-soluble compounds. This fact renders the isolation of pure labeled choline and phosphocholine by thin-layer chromatography impossible.

3.3.6 Mice homozygous for the disruption of Pempt2 display no obvious physiological defects

Mice homozygous for the disrupted *Pempt2* allele displayed no obvious pathophysiological or behavioral defects, nor did they appear to have a reduced life span or altered body weight. Breeding ability of *Pempt2*(-/-) was indistinguishable from *Pempt2*(+/+) and (+/-) mice. Histological examination of liver sections from adult mice revealed no obvious changes in hepatocyte morphology. In preliminary examinations of animals maintained under normal laboratory conditions, there were no significant changes in lipoprotein profile, nor in plasma triacylglycerol, cholesterol or phospholipid levels. Finally, there were no significant differences in the bile acid or phospholipid concentrations in the bile, nor in the volume of bile collected from the gallbladder (data not shown).

3.4 Discussion

Using well-established gene targeting techniques, we generated mice carrying a disrupted allele of the *Pempt2* gene. This represents the first application of the "knockout" mouse technique to a gene for phospholipid biosynthesis. Mice carrying two copies of the disrupted allele lacked not only PEMT2, but virtually all methylation activity against the lipid substrate intermediates PMME and PDME. This result indicates that we have also eliminated PEMT1, the endoplasmic reticulum form of PEMT. Therefore, PEMT1 and PEMT2 are encoded by the same gene, *Pempt2*. The difference between these two forms of PEMT, which results in their divergent subcellular localizations, must be generated post-transcriptionally. Possible differences in post-transcriptional and post-translational modifications between PEMT1 and PEMT2, including RNA editing, alternative splicing, phosphorylation, glycosylation and fatty acylation, will be the focus of future study. Normal mice incorporated [L-*methyl*-^{3}H]methionine into PC within 5 min of tail vein injection. However, mice homozygous for the null allele had a greatly reduced ability (<5% of wild-type) to incorporate [L-*methyl*-^{3}H] methionine into choline-containing phospholipids, further confirming the elimination of PEMT activity. Mice heterozygous for the null allele had approximately 50% of the PEMT activity of normal mice, suggesting a gene dosage effect, without increased expression of the remaining functional copy of the *Pempt2* gene as compensation.

Mice homozygous for the disruption of *Pempt2* retained a low level of methylation activity against the initial lipid substrate PE. Therefore, it appears that a second PE methylation activity might exist in mice, with lower activity against PMME and PDME. We are investigating whether this activity is responsible for the

radiolabel associated with PC generated in *Pempt2*(-/-) mice following [L-*methyl*-^{3}H]methionine injection. Whether or not this PE methylation activity is authentic, it is quantitatively insignificant.

Mice deficient in PEMT activity showed very little change in their liver phospholipid composition. Despite the fact that PE methylation has been hypothesized to generate between 20 and 40% of liver PC (9), the *Pempt2*(-/-) mice showed no significant change in either PC (nmol/μg protein) or PC contribution to total liver phospholipid, compared to normal mice. PE, the initial substrate for methylation, was slightly elevated in the livers of mice lacking PEMT activity. Thus, either the PEMT contribution to total liver PC levels has been overestimated, or the CDP-choline pathway is stimulated to compensate for the loss of PEMT activity and to maintain hepatic PC levels. This latter hypothesis was tested by measurement of the activity of CT, the rate limiting step in the CDP-choline pathway. Homozygous knockout mice had 60% higher CT activity bound to membranes compared to normal mice. Since the membrane-bound form of CT is the active form, we hypothesize that CT is stimulated to compensate for the loss of PEMT activity. Previous studies also demonstrate a reciprocal regulation of PEMT and CT activities (14,16). For example, induction of hepatocyte proliferation increases CT activity while decreasing PEMT activity.

Radiolabeled methionine injected into the bloodstream was incorporated into PC in the liver of a normal mouse within as short a time as 5 min. After 24 h, the radiolabeled choline generated by PE methylation was distributed throughout the body of a normal mouse, showing that PEMT pathway can serve as a source of PC, not just for the liver, but for other tissues as well. However, it remains to be determined whether this PC is carried to other tissues as PC on the surface of

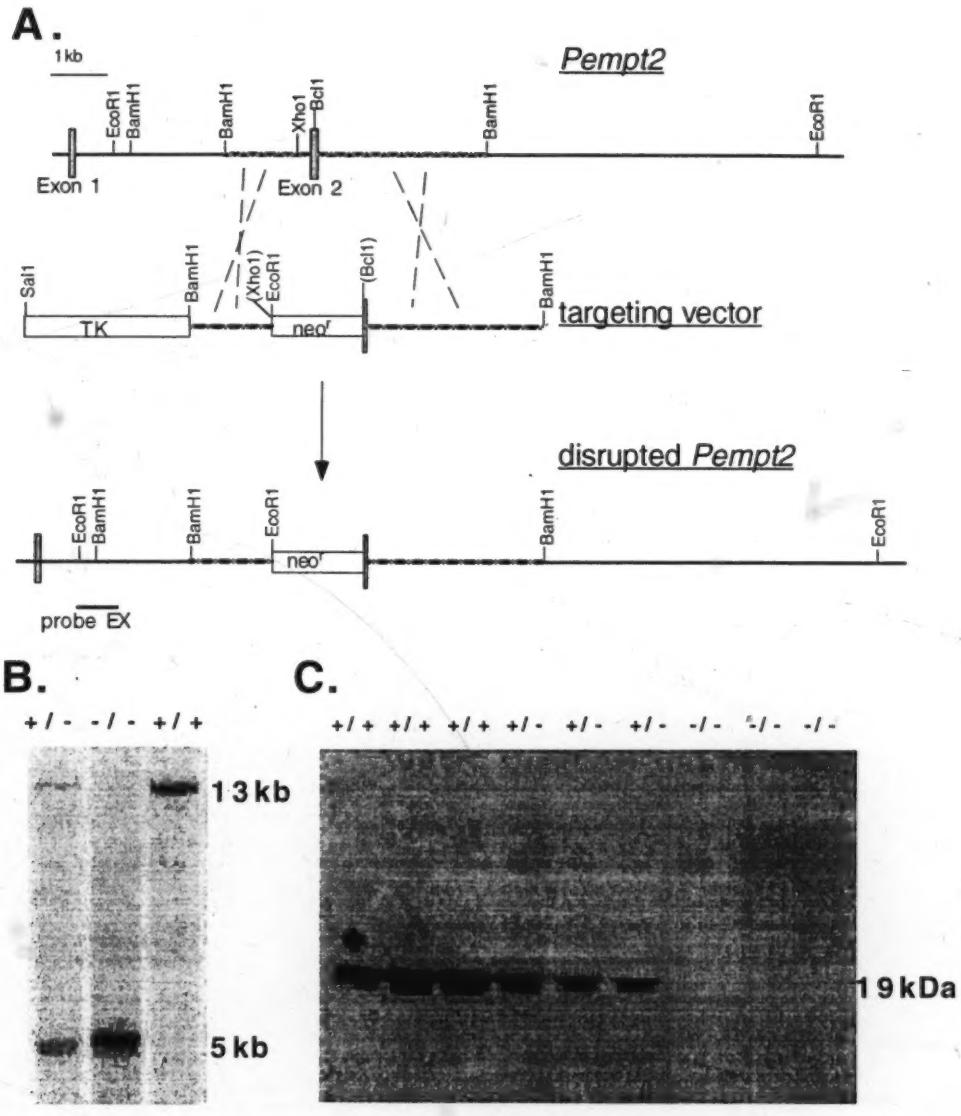
lipoproteins, or as lysoPC, or is distributed to other tissues as choline itself, and subsequently incorporated into PC via the CDP-choline pathway. PC could be secreted from the liver directly following its synthesis by PEMT, or generated from the catabolism of PE-derived PC to choline or phosphocholine, which is used for PC synthesis via the Kennedy pathway.

Mice lacking PEMT that were fed a normal chow diet did not display any abnormal phenotype. Therefore, it appears that the lack of PEMT can be compensated. We have demonstrated that CT activity was increased in the homozygous knockout mice, suggesting that the CDP-choline pathway substitutes for PEMT, and that the PC generated by this pathway is functionally equivalent to PC generated by PEMT. In other words, if PEMT-derived PC were the source of PC in mormal mice for a particular cellular function, for example lipoprotein and bile secretion, PC from the Kennedy pathway appears to be able to fulfill the same roles in *Pempt2*(-/-) mice. The question of whether PC generated by the two pathways is functionally equivalent has been previously studied. Rodents fed a diet deficient in choline survive, apparently because PC and choline are generated by PEMT, whose expression in the liver is increased (27). As well, hepatoma cell lines that completely lack PEMT expression, and primary hepatocytes exposed to PEMT inhibitors, survive with PC generated via the CDP-choline pathway (6,28). Conversely, Chinese hamster ovary cells carrying a lethal temperature-sensitive mutation in the CT gene cannot be rescued by overexpression of PEMT2 cDNA, despite the restoration of PC levels (29). Therefore, although CT can compensate for the loss of PEMT, the converse does not appear to be true: PEMT cannot compensate for the loss of CT.

The availability of these mice will allow us to address a number of other issues with respect to PEMT and its metabolic function. 1) What would the phenotype be if the mice were fed a choline-deficient diet? 2) Can dimethylethanolamine or monomethylethanolamine substitute for choline in PEMT-deficient mice? 3) Is the conversion of choline to betaine or acetylcholine altered in PEMT-deficient mice?

Figure 3.1 ***Pempt2* targeting strategy**

(A) The normal *Pempt2* gene, the gene targeting vector, and the disrupted *Pempt2* allele. The disrupted allele was identified by DNA blot analysis of *EcoR1*-digested DNA, using the 5' probe EX. *(B)* Genotyping of mice by DNA blot analysis. The normal *Pempt2* allele yielded a 13 kb EcoR1 fragment detected by probe EX, while the disrupted allele yielded a 5 kb EcoR1 fragment. *(C)* Absence of PEMT2 protein in *Pempt2* knockout mice. Immunoblot analysis of liver homogenates demonstrated that PEMT2 protein is present only in mice carrying at least one normal *Pempt2* allele. 50 μg protein/lane. PEMT2 has an apparent molecular mass of 19 kDa.


A.
1kb
Pempt2
EcoR1
BamH1
BamH1
Xho1
Bcl1
BamH1
EcoR1
Exon 1
Exon 2
Sal1
BamH1
(Xho1)
EcoR1
(Bcl1)
BamH1
TK
neo'
targeting vector
disrupted Pempt2
EcoR1
BamH1
BamH1
EcoR1
neo'
BamH1
EcoR1
probe EX
B.
+/- -/- +/+
13kb
5kb
C.
+/+ +/+ +/+ +/- +/- +/- -/- -/- -/-
19kDa

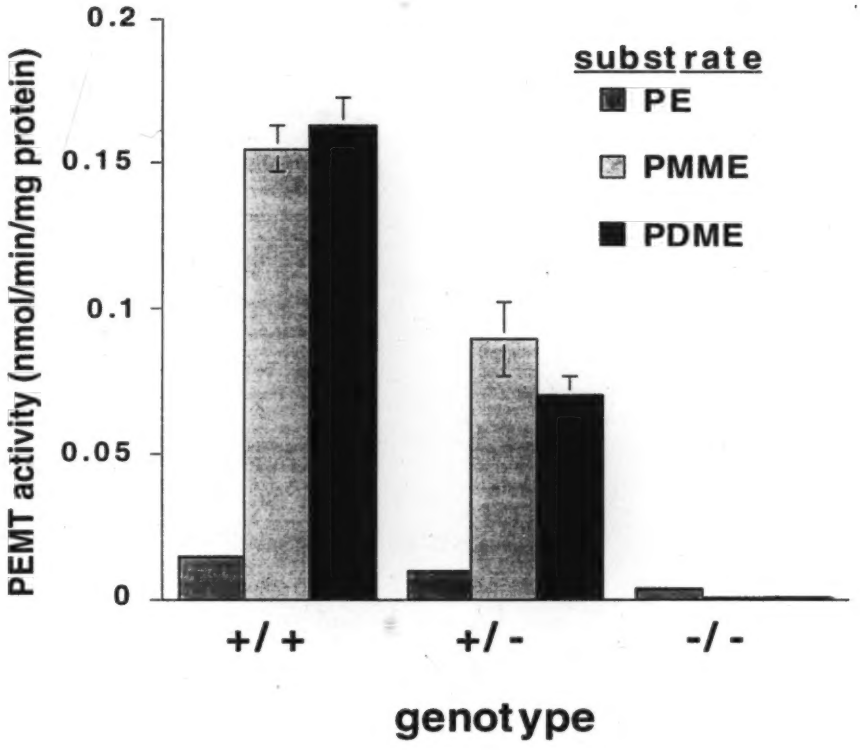


Figure 3.2 **Elimination of methylation activity of PE, PMME and PDME in *Pempt2* -disrupted mice**

Total liver homogenates from mice of all three genotypes were assayed for PEMT activity. n=4 for all three genotypes. Error bars represent standard deviations.

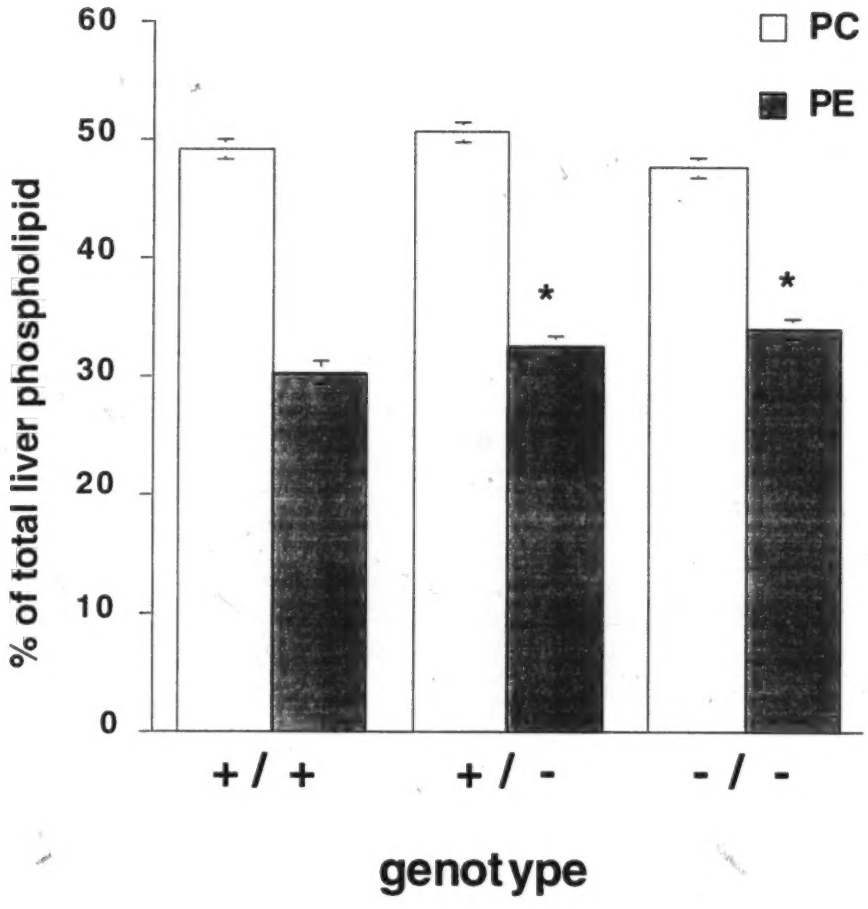


Figure 3.3 **Phospholipid composition of livers from mice of all three genotypes**

Levels of PC and PE in liver homogenates were measured and expressed relative to total phospholipid. n=4 in all cases. Error bars represent standard deviations. * $P<0.05$ compared to normal.

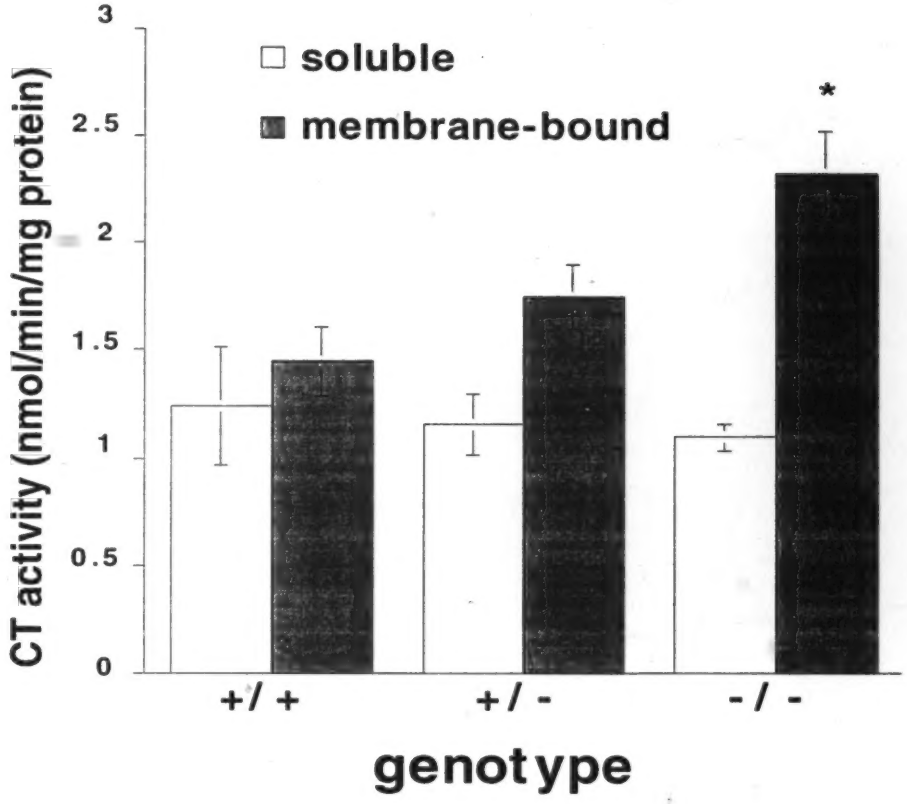
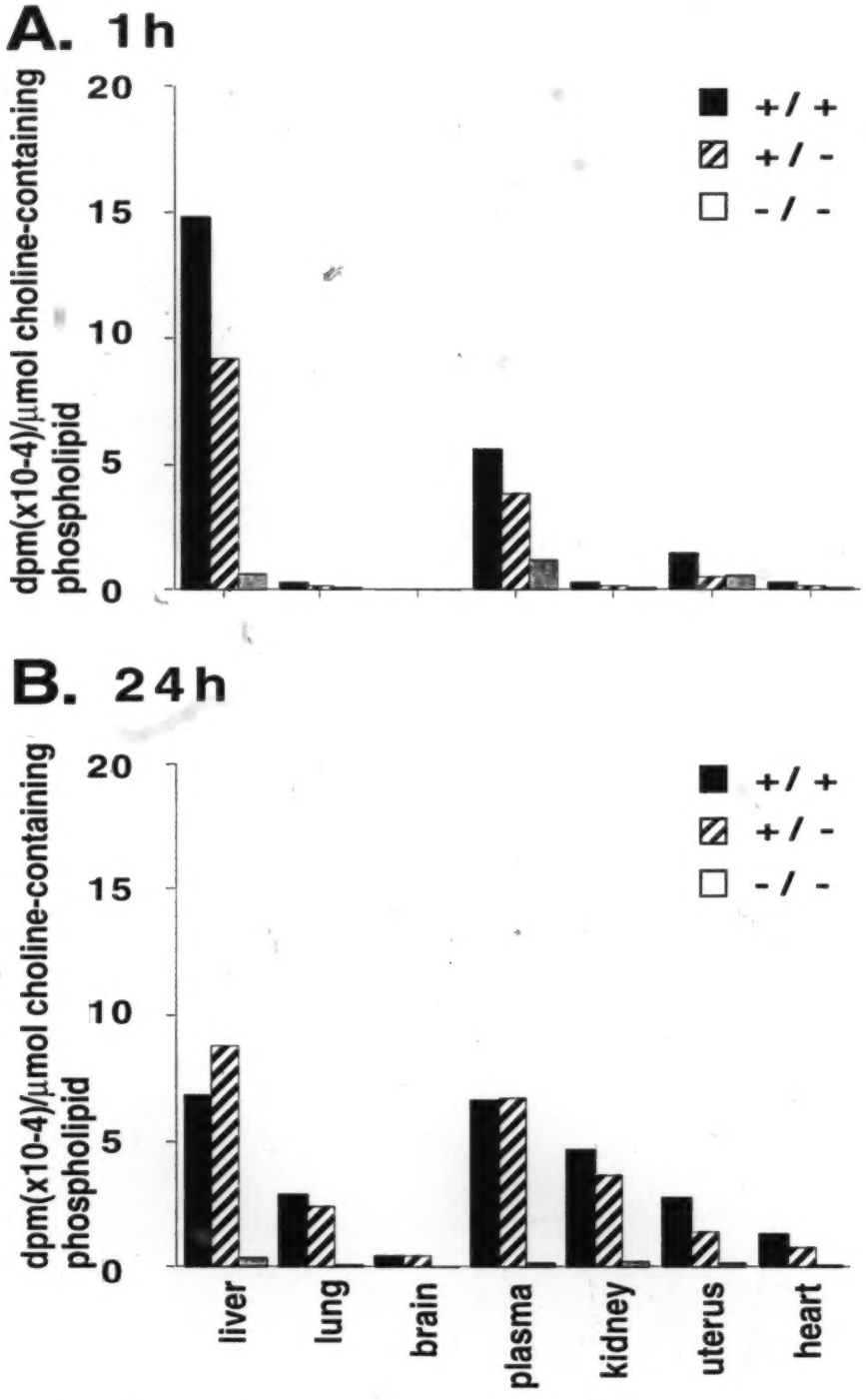


Figure 3.4 **Membrane-bound CT activity was increased in *Pempt2* knockout mice**

CT activity was measured in soluble and membrane-bound fractions of liver homogenates. n=4 in all cases. Error bars represent standard deviations. *$P<0.05$ compared to normal.

Figure 3.5 **Incorporation of radiolabeled methionine into choline-containing phospholipids**

[L-*methyl*-^{3}H]methionine was injected into the bloodstream of mice of each of the three genotypes. Plasma and organs were harvested after *(A)* 1 h or *(B)* 24 h. Phospholipids were extracted and separated by thin-layer chromatography. Bands corresponding to choline-containing phospholipids (PC, lysoPC and sphingomyelin) were scraped, and radiolabel incorporation measured, as well as total lipid phosphorous. Results are expressed as dpm per µmol choline-containing phospholipid. The mouse homozygous for the disrupted *Pempt2* allele had only 4.4% the level of radiolabeled methionine incorporation into choline-containing phospholipid seen in the normal mouse after 1 h, and 4.9% the level of incorporation after 24 h.


A. 1h
dpm(x10-4)/µmol choline-containing phospholipid
20
15
10
5
0
+/+
+/-
-/-
B. 24h
dpm(x10-4)/µmol choline-containing phospholipid
20
15
10
5
0
+/+
+/-
-/-
liver
lung
brain
plasma
kidney
uterus
heart

References

1. Kennedy, E. (1989) in *Phosphatidylcholine Metabolism* (Vance, D. E., ed), pp. 1-8, CRC Press, Boca Raton
2. Bremer, J., and Greenberg, D. M. (1961) *Biochimica et Biophysisca Acta* **46,** 205-216
3. Ridgway, N. D. (1989) in *Phosphatidylcholine metabolism* (Vance, D. E., ed), pp. 103-120, CRC Press, Boca Raton
4. Kodaki, T., and Yamashita, S. (1987) *Journal of Biological Chemistry* **262**(32), 15428-15435
5. Ridgway, N. D., and Vance, D. E. (1987) *Journal of Biological Chemistry* **262**(35), 17231-17239
6. Cui, Z., Vance, J. E., Chen, M. H., Voelker, D. R., and Vance, D. E. (1993) *Journal of Biological Chemistry* **268**(22), 16655-16663
7. Vance, J. E. (1990) *Journal of Biological Chemistry* **265**(13), 7248-56
8. Walkey, C. J., Cui, Z., Agellon, L. B., and Vance, D. E. (1996) *Journal of Lipid Research* **37**(11), 2341-2350
9. Sundler, R., and Akesson, B. (1975) *Journal of Biological Chemistry* **250**(9), 3359-3367
10. Vance, J. E., Nguyen, T. M., and Vance, D. E. (1986) *Biochimica et Biophysica Acta* **875**(3), 501-509
11. Vance, J. E., and Vance, D. E. (1986) *FEBS Letters* **204**(2), 243-246
12. Vance, J. E., and Vance, D. E. (1986) *Journal of Biological Chemistry* **261**(10), 4486-4491
13. Nishimaki-Mogami, T., Suzuki, K., and Takahashi, A. (1996) *Biochimica et Biophysica Acta* **1304**(1), 21-31

14. Houweling, M., Cui, Z., Tessitore, L., and Vance, D. E. (1997) *Biochimica et Biophysica Acta* **1346**(1), 1-9

15. Cui, Z., Shen, Y. J., and Vance, D. E. (1997) *Biochimica et Biophysica Acta* **1346**(1), 10-16

16. Tessitore, L., Cui, Z., and Vance, D. E. (1997) *Biochemical Journal* **322**(Pt 1), 151-154

17. Doetschman, T. C., Eistetter, H., Katz, M., and Kemler, R. (1985) *Journal of Embryology and Experimental Morphology* **87,** 27-45

18. Hogan, B., Beddington, R., Costantini, F., and Lacy, E. (1994) *Manipulating the Mouse Embryo: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Lab. Press, Plainview, NY

19. Laemlli, U. K. (1970) *Nature* **227,** 680-685

20. Ridgway, N. D., and Vance, D. E. (1992) *Methods in Enzymology* **209,** 366-374

21. Yao, Z. M., Jamil, H., and Vance, D. E. (1990) *Journal of Biological Chemistry* **265**(8), 4326-4331

22. Bligh, E. G., and Dyer, W. J. (1959) *Canadian Journal of Biochemistry and Physiology* **37,** 911-917

23. Bartlett, G. R. (1959) *Journal of Biological Chemistry* **234,** 466-468

24. Tijburg, L. B. M., Geelen, M. J. H., and van Golde, L. M. G. (1989) *Biochimica et Biophysisca Acta* **1004,** 1-19

25. Vance, D. E. (1990) *Biochemistry & Cell Biology* **68**(10), 1151-1165

26. Kent, C. (1990) *Progress in Lipid Research* **29**(2), 87-105

27. Cui, Z., and Vance, D. E. (1996) *Journal of Biological Chemistry* **271**(5), 2839-2843

28. Nishimaki-Mogami, T., Suzuki, K., Okochi, E., and Takahashi, A. (1996) *Biochimica et Biophysica Acta* **1304**(1), 11-20

29. Houweling, M., Cui, Z., and Vance, D. E. (1995) *Journal of Biological Chemistry* **270**(27), 16277-16282

Chapter 4

Choline-deficiency causes lethal liver failure in mice lacking PE methylation

An abridged version of this chapter will be submitted as a manuscript to *Science*.

4.1 Results and Discussion

Phosphatidylcholine (PC) is the most abundant phospholipid in eukaryotes, serving both as a structural component of membranes, and as a source of intracellular second messengers. Its importance is highlighted by the fact that there is no known disease where PC biosynthesis is impaired, suggesting that its absence is lethal very early in embryonic development. All eukaryotic cells use choline as a precursor for the synthesis of phosphatidylcholine (PC) via the CDP-choline pathway. The mammalian liver contains a second pathway for the synthesis of PC: three transmethylations of phosphatidylethanolamine (PE). These reactions are catalyzed by the enzyme phosphatidylethanolamine *N*-methyltransferase (PEMT).

Feeding rats and mice a choline-deficient (CD) diet is not lethal, despite impairment of the net biosynthesis of PC via the CDP-choline pathway. Hepatic PC levels are only slightly reduced in rats fed a CD diet, suggesting a compensatory mechanism (1-3). The most obvious candidate is the methylation of PE. In fact, expression of PEMT is stimulated during choline-deficiency in rats (2-5). This pathway generates not only PC, but also choline that can be utilized by the CDP-choline pathway. PEMT is believed to be the only endogenous source of choline, contributing approximately 15% of the body's daily requirement (6). We have previously found that in mice, choline generated from the methylation of PE in the liver is distributed throughout the body (7). Therefore, PEMT may serve to maintain PC levels not only in the liver but throughout the whole animal in the absence of dietary choline.

We have constructed mice that lack PEMT activity by targeted disruption of the *Pempt* gene (7). On a standard laboratory chow diet, these mice display no phenotypic abnormalities, suggesting that the CDP-choline pathway compensates for the lack of PEMT. This chapter examines the effects of feeding *Pempt*(-/-) mice a diet lacking choline. In this way, both major pathways for the biosynthesis of PC will be inhibited. Therefore, we will be able to determine whether PEMT functions as a compensatory pathway for the biosynthesis of PC in the absence of dietary choline. As well, we will be able to examine the physiological effects of a complete inhibition of net PC biosynthesis on the whole animal.

Eight-week-old mice of all three genotypes were placed on a CD or a choline-supplemented (CS) diet for three days. A significant weight loss was observed in *Pempt*(-/-) mice on a CD diet (Fig. 4.1). At this point, these *Pempt* (-/-) mice appeared sluggish and uncoordinated, and all the animals were sacrificed. The *Pempt*(-/-) mice on a choline-deficient diet had a grossly enlarged liver, with a striking white color suggesting massive fat accumulation (Figs. 4.2). The *Pempt*(+/+) mice on a CD diet also showed some fatty liver development as expected (8-10), although to a much lesser extent than seen in the *Pempt*(-/-) mice. On a CS diet, no abnormality was observed on liver morphology of any of the mice. A microscopic examination of liver sections from *Pempt* (-/-) mice fed a CD diet revealed a high degree of vacuolization and hemorrhaging (Fig. 4.3). A much lesser degree of vacuolization was also observed in the normal and heterozygous mice on a CD diet. No significant vacuolization was observed in mice of any genotype on the CS diet. To determine whether the vacuolization was a consequence of fat accumulation, liver sections from the mice were stained with Oil Red O (Fig. 4.4). Intense

staining was observed in sections from *Pempt*(-/-) mice fed a CD diet (bottom right), confirming the massive fat accumulation in the livers of these mice.

The phospholipid composition of the liver from mice with and without PE methylation activity and fed a CD diet was investigated. *Pempt* (-/-) displayed a greatly reduced level of hepatic phospholipid (Fig. 4.5A). Total liver phospholipid was decreased from 125.4 nmol/mg protein in *Pempt*(-/-) fed a CS diet to 73.2 nmol/mg protein in *Pempt*(-/-) mice on a CD diet, a 41.6% decrease. In contrast, the phospholipid level in the liver was not significantly different between normal mice with full PEMT activity fed a CD or CS diet. Hepatic PC levels were also greatly reduced in *Pempt*(-/-) animals on a CD diet (Fig. 4.5B). PC content in the liver fell from 55.1 nmol/mg protein in normal mice fed a CD diet to 27.6 nmol/mg protein in PEMT-deficient mice fed a CD diet, a drop of 50.0%. Normal mice also experienced a slight drop in hepatic PC levels on a CD diet. A decrease of equal magnitude has been previously observed in rats fed a CD diet, but not to nearly the extent observed in the PEMT-deficient mice(11). Thus, in *Pempt*(-/-) mice on a CD diet, PC accounted for less than 38% of hepatic phospholipid, compared to 50% or greater in all other animals on either diet (Fig. 4.5C).

The activity of CTP:phosphocholine cytidylyltransferase (CT), which catalyzes the rate-limiting step of the CDP-choline pathway for PC biosynthesis, was measured. CT translocates between an inactive soluble form and an active membrane-bound form (12). Translocation to the membrane-bound form, and hence activation of CT, has been previously observed in the livers of rats fed a CD diet (13). A similar translocation was observed in these studies: in normal mice, membrane-bound CT activity was increased 57.9% ($P<0.05$), from 1.49

nmol/min/mg protein to 2.36 nmol/min/mg protein (Fig. 4.6C). Total CT activity in normal mice was also increased 27.8% ($P<0.05$), from 2.41 nmol/min/mg protein on a CS diet to 3.08 nmol/min/mg protein on a CD diet, suggesting that perhaps CT gene expression is stimulated by choline-deficiency (Fig. 4.6A). Similar changes were also observed for mice heterozygous for the null *Pempt* allele, where translocation of CT to the membrane-bound form was even further stimulated compared to the normal mice. This response to choline-deficiency may represent an adaptation designed to maximize utilization of available choline. Unlike mice fed a standard laboratory chow diet, *Pempt*(-/-) mice fed a CS diet did not exhibit an elevated membrane-bound CT activity compared to their normal siblings. The reason for this discrepancy may lie in the differences between the natural lab chow, and the synthetic CS diet, in particular the difference in fat content (9% in the lab chow compared to 20% in the CS diet). However, on a CD diet, hepatic membrane-bound CT activity was elevated 26.0% in *Pempt*(+/-) mice compared to normal mice , from 2.36 nmol/min/mg protein to 2.97 nmol/min/mg protein, and 29.7% in *Pempt*(-/-) mice, to 3.06 nmol/min/mg protein, although the statistical significance of this increase is questionable, due to large sample-to-sample variation on measurement. The *in vitro* activity of the soluble form of CT is unchanged in all the mice on both diets, except for *Pempt*(-/-) mice, where the activity falls more than 50% compared to other animals (Fig. 4.6B). This decrease may be caused by leakage of the soluble CT into the bloodstream as a consequence of liver damage (discussed below). The fact that total CT activity is slightly lower in the livers of these mice compared to their normal and heterozygous counterparts on the same diet also suggests loss of soluble CT. Such a leakage may disrupt the equilibrium between the soluble and membrane-bound forms of CT, preventing maximal activation of CT by translocation.

We also measured changes in neutral lipid composition of the livers of mice fed both diets. The level of triacylglycerol in the liver of *Pempt*(-/-) mice was increased 2.6-fold compared to normal mice fed a CD diet, and 2.1-fold compared to *Pempt* (-/-) mice fed a CS diet (Fig. 4.7). This result coincides with the histological examination of liver sections, where heavy vacuolization, presumably due to lipid accumulation, was observed in *Pempt* (-/-) fed a CD diet (Figs. 4.3 and 4.4). Despite the slight vacuolization observed in normal mice fed a CD diet, there was no significant difference in the liver TG values among mice containing PEMT activity fed either a CS or CD diet. This result is different from the situation observed in rats, where there is a rapid accumulation of triacylglycerol upon feeding of a CD diet (14). As well, hepatic free cholesterol was reduced in the livers of CD mice compared to CS mice, except for the *Pempt*(-/-) mice, where hepatic free cholesterol was elevated (Fig. 4.8).

Although detailed investigations were not performed, the morphology of other organs in *Pempt* (-/-) mice fed a CD diet appeared normal. PEMT-derived PC is found in other tissues, so why does the lack of PEMT plus choline-deficiency appear to affect primarily the liver? First, in the liver (and kidney), pre-existing choline can be incorporated into betaine, reducing the amount available for PC biosynthesis. Betaine is a substrate for the biosynthesis of methionine and thus AdoMet. However, without PE methylation betaine-derived AdoMet does not contribute to the generation of new choline. Second, the liver contains two PC-requiring functions: the synthesis and secretion of very low-density (VLDL) and high-density (HDL) lipoproteins, and the secretion of bile. These functions, not present in other tissues, drain hepatic PC stores. The

effects of the elimination of net PC biosynthesis by *Pempt* disruption and choline-deficiency on these functions are investigated below.

Phosphatidylcholine is an important component of lipoproteins, forming a monolayer around the neutral lipid core of the particles, thus enhancing their solubility in plasma. The fatty liver observed in rats fed a CD diet is at least partially due to a defect in the secretion of triacylglycerol-rich VLDL, resulting in hepatic triacylglycerol accumulation (11,15). Therefore, the biosynthesis of PC is a crucial component of VLDL secretion. We were interested in determining the plasma lipid levels in mice lacking the ability to convert PE to PC. Plasma was isolated from mice of all three genotypes fed either a CS or CD diet, and triacylglycerol levels measured (Fig. 4.9). *Pempt*(-/-) mice fed a CD diet exhibited a lower total plasma triacylglycerol level (measured as glycerol) than normal mice fed a CD diet. Interestingly, mice heterozygous for the null *Pempt* allele and fed a CD diet also displayed decreased plasma triacylglycerol levels. These mice have approximately 50% the PEMT activity of their normal siblings. It is important to note that we measured 'steady-state' lipid levels, and could not determine whether the changes we observed were due defects in lipoprotein secretion or stimulation of lipoprotein uptake. Unlike the case in rats, normal mice did not display a lower plasma triacylglycerol level when fed a CD diet compared to a CS diet (16,17). Plasma cholesterol levels were also measured (Fig 4.10). On a CS diet, plasma cholesterol levels were slightly lower in *Pempt*(+/-) and (-/-) mice compared to (+/+) mice. This effect was enhanced in mice fed a CD diet, where plasma cholesterol levels decreased 79%, from 4.08 mM in normal mice to 0.86 mM in *Pempt*(-/-) mice. Finally, on a CS diet, plasma phospholipid levels were reduced 36% in *Pempt*(-/-) mice compared to their normal counterparts, from 2.52 mM to 1.62 mM (Fig. 4.11). However, on a CD

diet, this variation according to genotype was enhanced. Plasma phospholipid levels fell from 1.88 mM in normal mice to 0.56 in *Pempt*(-/-) mice, a drop of 70%. Compared to their CS counterparts, PEMT-deficient CD mice had only 34% the level of plasma phospholipid. These results strongly suggest that elimination of PE methylation combined with choline-deficiency greatly affects lipoprotein metabolism.

In order to more closely examine the role of dietary choline and PEMT activity on the lipoprotein profiles of mice, plasma from mice on a CD diet was pooled according to genotype, and individual classes of lipoproteins separated by high performance liquid chromatography (HPLC). The relative amounts of triacylglycerol and cholesterol in each fraction was determined (Fig. 4.12). As expected, the majority of plasma triacylglycerol from normal mice was found in the VLDL fraction. Plasma from mice heterozygous for the null *Pempt* allele had a lower amount of triacylglycerol in the VLDL fraction, a result that matches the measurement of total plasma triacylglycerol from above. *Pempt*(-/-) mice displayed very little triacylglycerol in the VLDL fraction. Instead, triacylglycerol accumulated in a fraction with the same size as low-density lipoprotein (LDL). Whether this fraction is in fact apolipoprotein B-containing LDL, or another kind of plasma lipid particle of a similar density, notably lipoprotein X observed during cholestasis (18), remains to be determined. In normal mice, the majority of plasma cholesterol was found in the HDL fraction, as expected. Just as for total plasma cholesterol, *Pempt*(+/-) had slightly reduced levels of HDL cholesterol compared to normal mice. More strikingly, *Pempt*(-/-) mice had greatly reduced levels of HDL cholesterol. These results demonstrate the drastic effects that inhibition of PC biosynthesis, caused by both choline deficiency and elimination of PE methylation, have on lipoproteins, and thus

reinforce the importance of PC biosynthesis for lipoprotein metabolism. However, whether the reduction in circulating lipoproteins is due to a decrease in secretion caused by the lack of PC biosynthesis, or to a stimulation of the LDL receptor to increase the uptake of lipoproteins remains to be determined. Both explanations are attractive. In isolated rat hepatocytes the active synthesis of PC is required for VLDL secretion (15). Therefore, the accumulation of lipid in the livers of *Pempt* (-/-) mice fed a CD diet may be a result of the impairment of VLDL secretion. This hypothesis is best tested on isolated hepatocytes, where changes in the secretion of radioactive precursors can be observed. Conversely, uptake of lipoproteins may be stimulated in order to increase cellular PC levels. It may be that the observed changes in plasma lipoprotein levels may be a consequence of both of these factors.

PC is also an important component of bile, where it forms micelles with bile acids that aid in the absorption of dietary fats. PC is secreted from hepatocytes into the bile caniculi of the liver along with other components of bile, and collected in the gall bladder. Figure 4.2 shows massive accumulation of bile in the gall bladders of *Pempt*(-/-) fed a CD diet. Measurement of the volume of bile collected from mice of all genotypes confirmed this observation (Fig. 4.13). The largest volumes of bile were collected from *Pempt*(-/-) mice fed a CD diet. Two additional effects were observed. First, in mice fed a CS diet, bile volume increased as PEMT activity decreased from *Pempt*(+/+) mice to *Pempt*(+/-) mice to *Pempt*(-/-) mice. This result suggests that PE methylation plays an important role in bile metabolism i. e. is inhibitory to the emptying of the gall bladder, independent of choline-deficiency. However, a similar pattern was not observed in mice of different genotypes fed a standard laboratory diet. As well, normal mice, but not mice heterozygous for the null allele, had larger bile

volumes in their gall bladders when fed a CD diet than when fed a CS diet, suggesting that choline-deficiency affects bile movement, even when the compensatory PEMT pathway is available.

The consequences of choline-deficiency and PE methylation on the composition of bile were also examined. No significant effect of either choline-deficiency or different PEMT activities on bile acid concentration was observed (Fig. 4.14). However, cholesterol concentration in the bile was decreased approximately 30% ($P<0.05$) when mice of all three genotypes were fed a CD diet compared to a CS diet (Fig. 4.15). The PC contribution to bile was measured in three ways. First, total bile phospholipid was determined (Fig. 4.16A). Since it makes up more than 95% of the phospholipid in bile, we believed that this would be an accurate method for estimation of PC levels. However, lipids were not extracted prior to the assay, meaning that phosphates other than those from phospholipids were measured. Therefore, the results obtained may not accurately reflect the phospholipid levels in the bile. As an alternative, total choline was measured in bile. Large sample-to-sample variation was observed (Fig. 4.16B). However, it appeared that bile choline was decreased in *Pempt*(-/-) mice fed a CD diet. It is important to note that leakage of phosphocholine into bile as a consequence of liver damage (discussed below), means that the measurement generated in this instance may overestimate the actual PC content. Perhaps the most convincing demonstration that the combination of choline-deficiency and lack of PE methylation significantly decreases bile PC levels is an examination of a thin-layer chromatography separation of bile components (Fig. 4.16C). The band corresponding to PC from CD, *Pempt*(-/-) bile was notably lighter than bands from other samples. No bands corresponding to other phospholipids were observed in any of the bile

samples, suggesting that other phospholipids are not secreted into bile to compensate for the lack of PC. Two explanations for the decrease in bile PC in *Pempt* (-/-) mice present themselves. The first is that the decrease is merely a consequence of the liver damage observed, resulting in an impairment of the PC secretion process. The second is that the decrease is an adaptation designed to conserve PC levels in the liver. Which explanation is the correct one remains to be determined. It is interesting to compare our bile phenotype to that generated by targeted disruption of the *mdr2* gene in mice, where PC in the bile is completely eliminated (19). In *mdr2* (-/-) mice, bile cholesterol is decreased 15-fold, suggesting coupling between PC and cholesterol secretion . However, in *Pempt* (-/-) fed a CD diet, bile cholesterol is unaffected while bile PC is reduced, but not eliminated. Therefore, it appears that the level of PC in the bile of *Pempt* (-/-) mice fed a CD diet, although greatly reduced, is sufficient for normal cholesterol secretion. *mdr2* (-/-) mice also show abnormal liver histology, although not the extensive vacuolization seen in *Pempt* (-/-) mice fed a CD diet. The liver damage observed in *mdr2* (-/-) mice is not lethal. Thus the phenotypes we observe in *Pempt* (-/-) mice are due to more than just an alteration in bile PC secretion.

Choline is a precursor of *S*-adenosylmethionine (AdoMet), a methyl group donor for several substrates, including PE (20). Therefore, we measured the consequences of choline-deficiency and the elimination of PEMT activity on the hepatic levels of AdoMet, and its de-methylated product, *S*-adenosylhomocysteine (AdoHcy). In all cases, choline-deficiency resulted in a drop of more than 50% in hepatic AdoMet concentration (Fig. 4.17A). AdoHcy levels were also decreased, though not to the same degree (Fig. 4.17B). Therefore, the ratio of AdoMet to AdoHcy was higher in CS mice (2.7-3.2) than

in CD mice (1.5-1.9) (Fig. 4.17C). This result most likely reflects a shortage of the choline precursor. Interestingly, *Pempt* genotype had no significant effect. This may be because PE methylation both consumes AdoMet, producing AdoHcy, while at the same time stimulating AdoMet production by generating its choline precursor, resulting in a minimal net change in hepatic AdoMet levels.

The drastic changes in liver morphology and lipid content, lipoprotein metabolism and bile trafficking strongly suggest that the combination of choline-deficiency and the elimination of PE methylation results in severe, and very likely lethal, liver damage. To investigate this hypothesis, we measured levels of alanine and aspartate aminotransferase in the plasma. These enzymes are normally present in the cytosol of hepatocytes, but leak out into the bloodstream when the liver is damaged. The level of alanine aminotransferase (ALT) activity in pooled plasma was increased 12.5-fold in *Pempt*(-/-) mice on a CD diet compared to normal mice on a CD diet (Table 4.1). *Pempt* (-/-) mice on a CS diet had an undetectable plasma ALT activity, while the same mice on a CD diet had a plasma ALT level of 417 IU/L. A similar pattern was observed when aspartate aminotransferase (AST) in the plasma was measured (Table 4.1). When liver damage occurs, plasma AST and ALT levels skyrocket, then fall again as further damage eliminates expression of these enzymes (21). Therefore, in these mice, liver damage after three days on the diets may be beyond this point. Measurement of ALT and AST at earlier points during feeding may give higher values from *Pempt* (-/-) mice fed a CD diet. As well, the level of bile acids in the plasma, another indicator of liver damage, was elevated 5.6-fold in *Pempt*(-/-) mice fed a CD diet compared to *Pempt* (+/+) mice fed a CD diet (Fig. 4.18). The plasma itself in these mice is bright yellow, suggesting severe jaundice. Clearly, a CD diet in the absence of PEMT activity resulted in

severe liver damage. As mentioned above, a visual examination of other tissues under these conditions did not reveal any obvious gross abnormalities.

The question of whether choline is an essential or dispensable nutrient has been long disputed (22). The fact that animals fed a choline-deficient diet survive, albeit with some deleterious effects, suggests that a compensatory endogenous pathway exists for the generation of choline. The best characterized of the possible pathways is the methylation of PE to PC. We have previously demonstrated that choline generated by PE methylation in the liver can be found throughout the body as PC (7). In the experiments described here, we have shown that the absence of PE methylation greatly amplifies the consequences of choline-deficiency, causing severe liver damage, altered lipoprotein metabolism and accumulation of bile in the gall bladder. These effects are almost certainly lethal to the animals. We hypothesize that the primary defect is the drop in hepatic phosphatidylcholine levels. This results in a disruption of membrane integrity and function, leading to a leakage a hepatocyte contents and an inability to secrete lipoproteins. Therefore, these results suggest that one function of PE methylation, which may explain its survival throughout evolution, is the endogenous synthesis of choline as well as PC, particularly in the absence of dietary choline. Under these conditions, the net biosynthesis of PC in the liver is necessary for the survival of the animal.

The relevance of combined PEMT- and choline-deficiency to humans in terms of PC biosynthesis is questionable. There is no known disease where PC biosynthesis is deficient, presumably because of its importance very early in embryonic development. Chinese hamster ovary cells carrying a temperature-sensitive mutation in the CDP-choline pathway die at the restrictive

temperature, further highlighting the importance of PC biosynthesis (23). The fact that on a standard diet, mice lacking PEMT are phenotypically indistinguishable from their normal littermates suggests that humans lacking PEMT would appear normal. As well, a CD diet is a laboratory phenomenon; virtually all foods contain at least some choline, primarily as PC. Choline-deficiency does occur during starvation, therefore, we will investigate fasting in mice with and without PEMT.

Nonetheless, the combination of PEMT- and choline-deficiency may be a valuable model for liver disease. Cholestasis is defined from a physiological point of view as a decrease of bile flow. Cholestasis can result from a variety of different causes, including hepatitis infection and alcoholism. While we have yet to measure bile flow in our mice, the *Pempt* (-/-) mice fed a CD diet display symptoms associated with cholestasis, most notably jaundiced plasma and elevated transaminases in the plasma, indicative of liver damage (24). However, unlike classical cholestasis, an elevation of plasma cholesterol and phospholipid levels does not occur in *Pempt* (-/-) mice fed a CD diet (25). As well, lipid accumulation is a symptom of some liver syndromes such as chronic alcoholism, but not others such as hepatitis. Nonetheless, for an investigator wishing to quickly generate liver dysfunction, feeding *Pempt* (-/-) mice a CD diet may be a valuable tool.

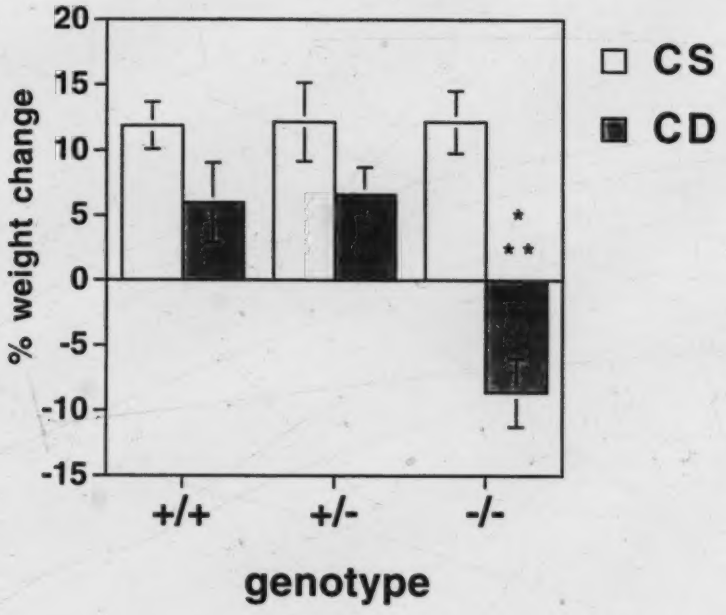


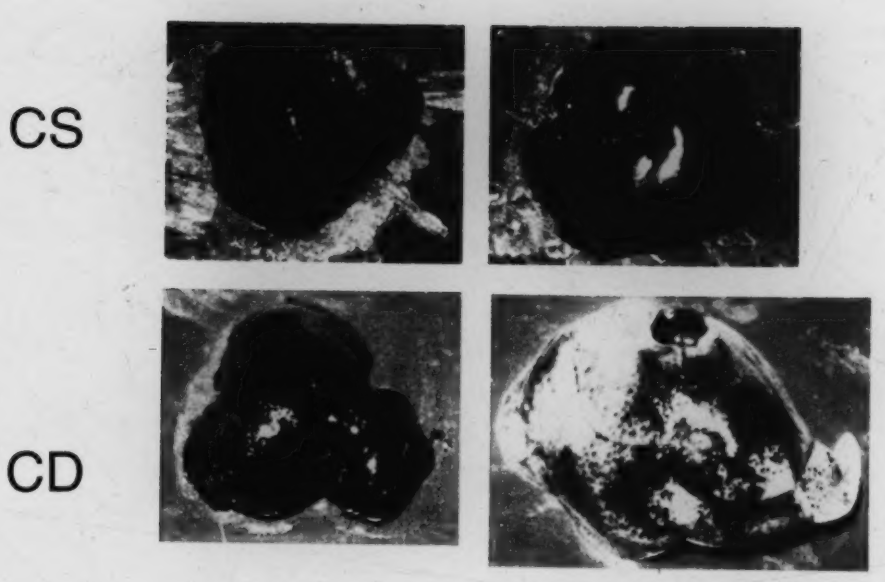
Figure 4.1 **Mice lacking PEMT fed a choline-deficient diet lost weight.**

Eight week-old mice of all three genotypes (*Pempt*(+/+), (+/-) and (-/-)), weighing between 16 and 28 g, were fed a choline-deficient (CD) diet (ICN, catalog # 901387) or a choline-supplemented (CS) diet (CD diet + 0.4% choline) for three days, then fasted for 12 hours and sacrificed. According to the manufacturer, each diet contains 20% fat as lard. The animals were weighed before and after the feeding, and the % weight change calculated. These mice were used for the analyses in the remainder of this chapter. n=3, 6, and 3 for *Pempt*(+/+), (+/-) and (-/-) mice respectively, fed a CS diet, . n=3, 8 and 5 for *Pempt*(+/+), (+/-) and (-/-) mice respectively, fed a CD diet. . The number of mice in all categories remains unchanged throughout the remainder of this chapter. *P< 0.05 compared to (+/+); **P< 0.05 compared to (+/-).

Figure 4.2 **Grossly enlarged liver in *Pempt*(-/-) mice fed a choline-deficient diet.**

(A) Feeding a CD diet to *Pempt*(-/-) mice for three days resulted in a massive enlargement of the liver (bottom right), most likely due to lipid accumulation, hence the white color and spongy texture. Note in particular the distended gall bladder at the top of the liver. The liver from normal mice fed a CD diet also showed signs of mild lipid accumulation, visible as a slight whitening (bottom left). Both *Pempt*(+/+) and (-/-) livers appeared normal following three days on a CS diet (top). (B) Livers were removed from the animals and weighed wet. In *Pempt*(-/-) mice on a CD diet for three days, the liver contributed over 6% (or >1.2 g) of the total body weight, compared to approximately 4% (or <1 g) in other animals. $^{*}P< 0.05$ compared to (+/+); $^{**}P< 0.05$ compared to (+/-).

A.



B.

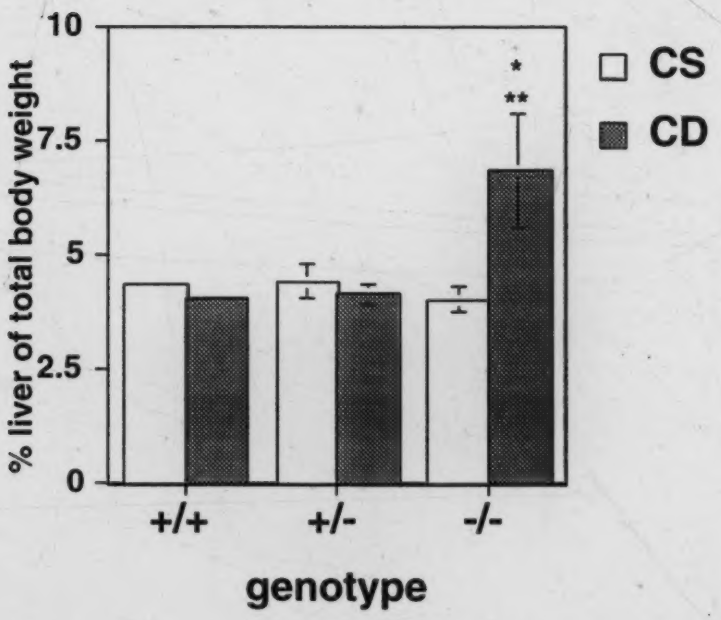


Figure 4.3 **Extensive vacuolization in liver sections from *Pempt*(-/-) mice fed a choline-deficient diet.**

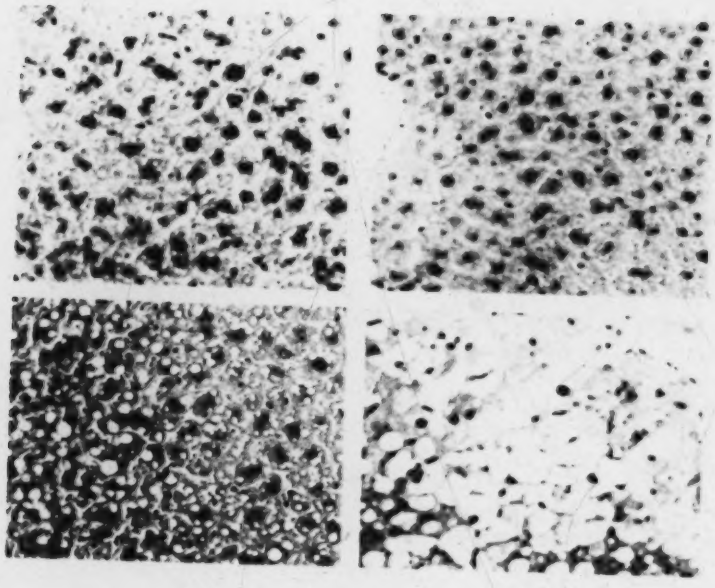
(A) Livers from mice fed a CD or CS diet were fixed in 10% formaldehyde, sectioned, and stained with hemotoxylin-eosin prior to microscopic examination at 200X magnification. Extensive vacuolization was observed in liver sections from *Pempt*(-/-) mice fed a CD diet (bottom right) as large white patches. Minor vacuolization was also observed in normal mice fed a CD diet (bottom left). No vacuolization was observed in any mice fed a CS diet. *Pempt* (+/-) mice closely resembled *Pempt* (+/+) mice under histological examination. (B) Another liver section from a *Pempt*(-/-) mouse fed a CD diet revealed extensive hemorrhaging, visible as darker red regions, suggesting massive liver damage.

A.

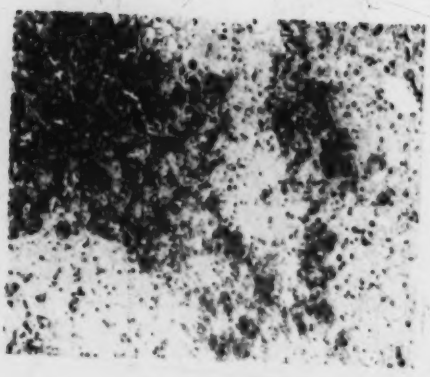
+/+ -/-

CS

CD



B.



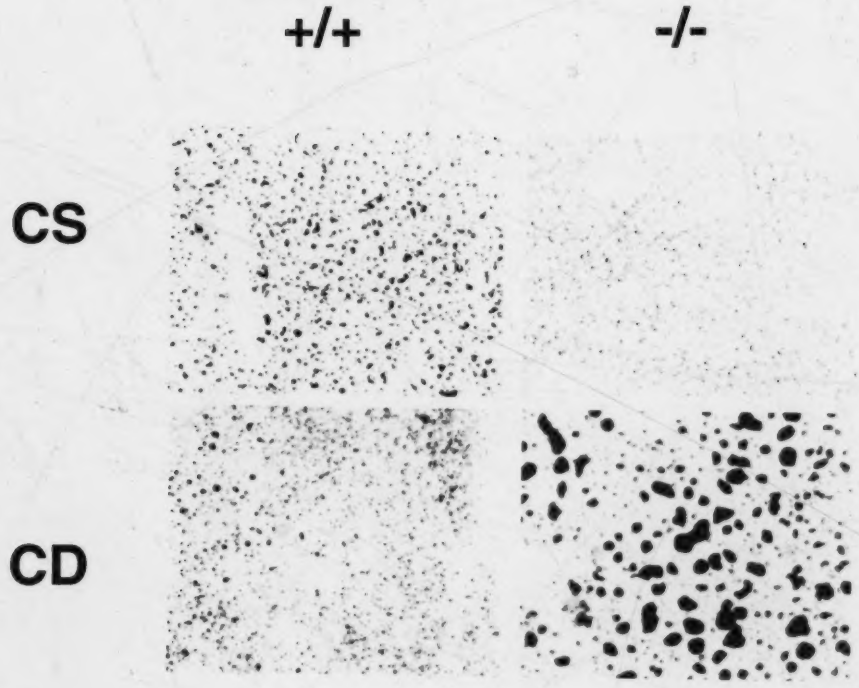


Figure 4.4 **Oil Red O staining of liver sections.**

Quick-frozen liver samples were sectioned and stained with lipophilic Oil Red O, then examined under 200X magnification for the presence of red patches, indicating lipid.

Figure 4.5 **Hepatic phospholipids, and particularly PC, were reduced in *Pempt*(-/-) mice fed a choline deficient diet.**

(A) Liver was homogenized according to standard procedure, and lipids extracted by the method of Bligh and Dyer (13). Lipid phosphorous was determined by the method of Bartlett (14), while protein concentration was determined by BCA assay (Pierce), according the kit manufacturer's instructions, and using bovine serum albumin as a standard. (B) PC was isolated from other phospholipids by thin-layer chromatography, and assayed (14). (C) The percentage of hepatic phospholipids that were PC was calculated, based on the above data. $^{*}P< 0.05$ compared to (+/+); $^{**}P< 0.05$ compared to (+/-).

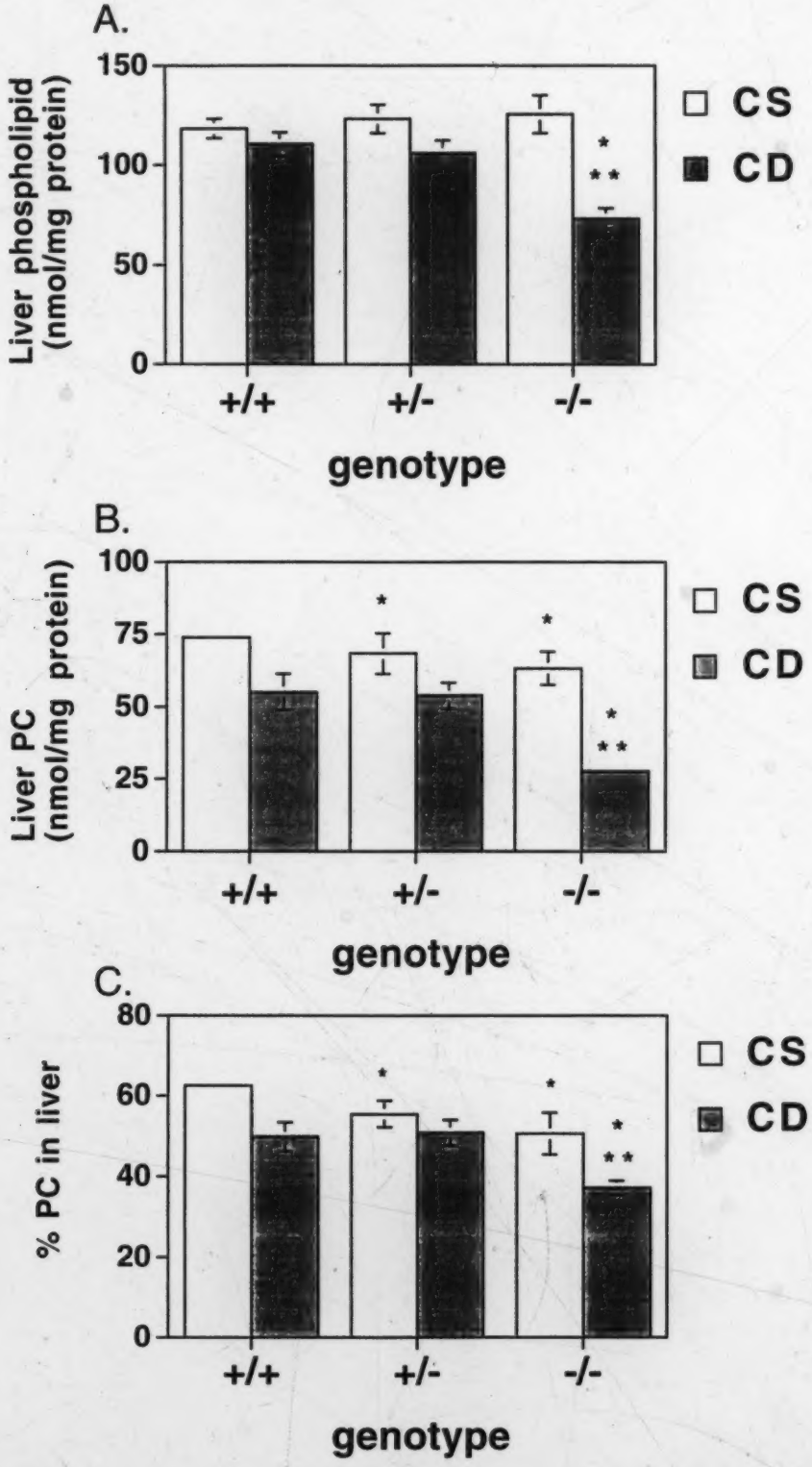
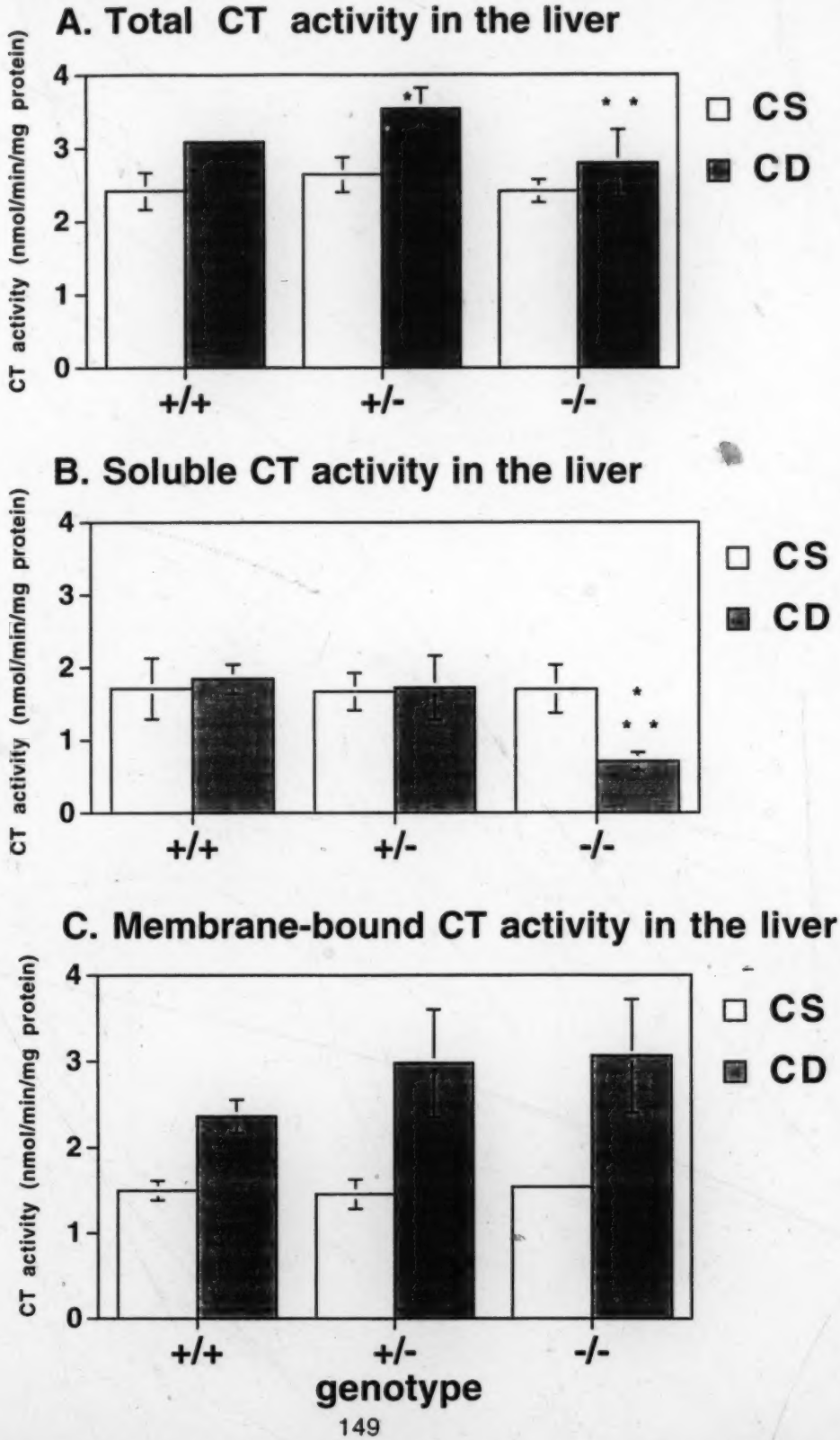

A.
Liver phospholipid (nmol/mg protein)
150
100
50
0
+/+
+/-
-/-
genotype
CS
CD
B.
Liver PC (nmol/mg protein)
100
75
50
25
0
+/+
+/-
-/-
genotype
CS
CD
C.
% PC in liver
80
60
40
20
0
+/+
+/-
-/-
genotype
CS
CD

Figure 4.6 **CT activity in choline-supplemented and choline-deficient mice.**

(A) CT activity was measured in total homogenates by standard methods (15). (B & C) Homogenates were fractionated into cytosol and microsomes by 100000xg centrifugation, and CT activity in each fraction measured Weinhold, 1986 #256 . *P< 0.05 compared to (+/+); **P< 0.05 compared to (+/-).


A. Total CT activity in the liver
CT activity (nmol/min/mg protein)
0
1
2
3
4
+/+
+/-
-/-
CS
CD
B. Soluble CT activity in the liver
CT activity (nmol/min/mg protein)
0
1
2
3
4
+/+
+/-
-/-
CS
CD
C. Membrane-bound CT activity in the liver
CT activity (nmol/min/mg protein)
0
1
2
3
4
+/+
+/-
-/-
genotype
CS
CD

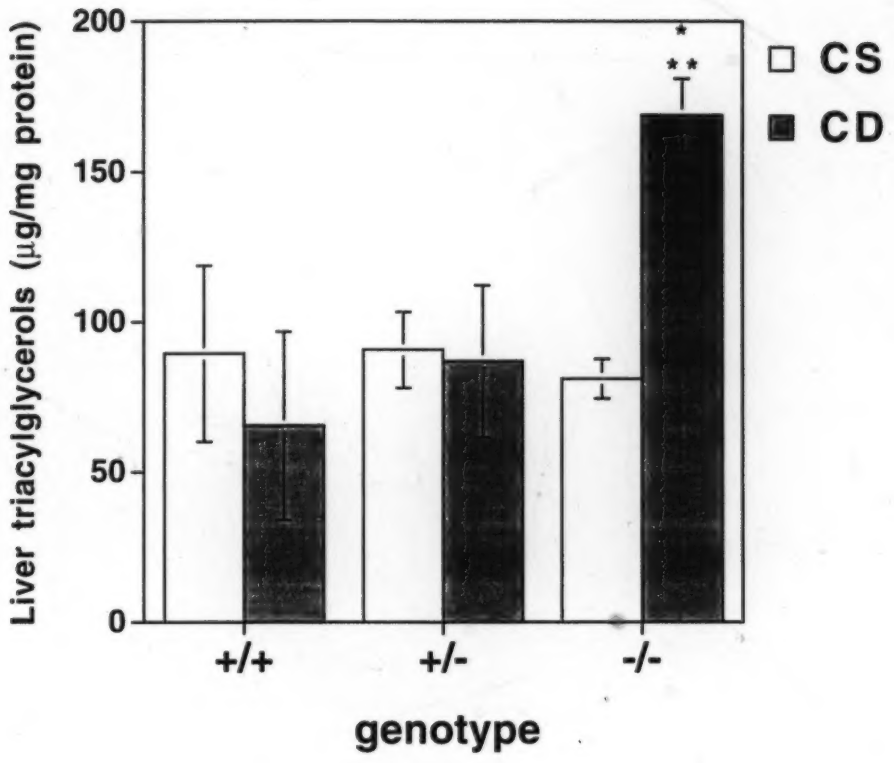


Figure 4.7 **Liver triacylglycerols**

Lipids were extracted from total liver homogenates, and triacylglycerols isolated by TLC, using hexane/diethyl ether/acetic acid (65/35/2) as a solvent. Bands corresponding to triacylglycerols were identified by comparison to a triolein standard following visualization by iodine vapour, and scraped. Triacylglycerol was assayed by the method of Snyder and Stephens, using TLC-purified triolein as a standard (26). *P< 0.05 compared to (+/+); **P< 0.05 compared to (+/-).

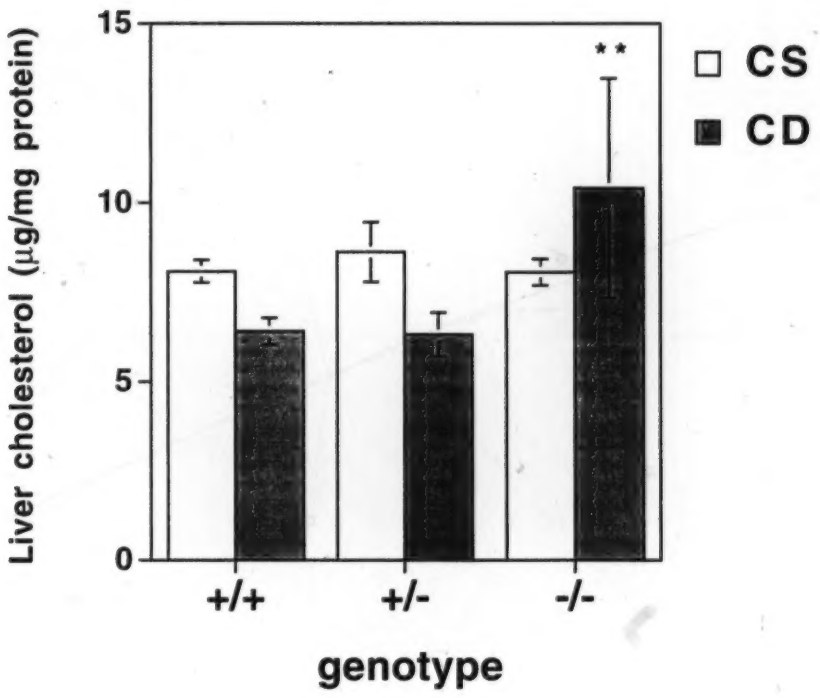


Figure 4.8 **Free cholesterol accumulates in the livers of *Pempt*(-/-) mice fed a choline-deficient diet.**

The amount of cholesterol in the livers of mice fed CD and CS diets measured using the Cholesterol CII kit (Wako), following the manufacturer's instructions. $^{**}P < 0.05$ compared to (+/-).

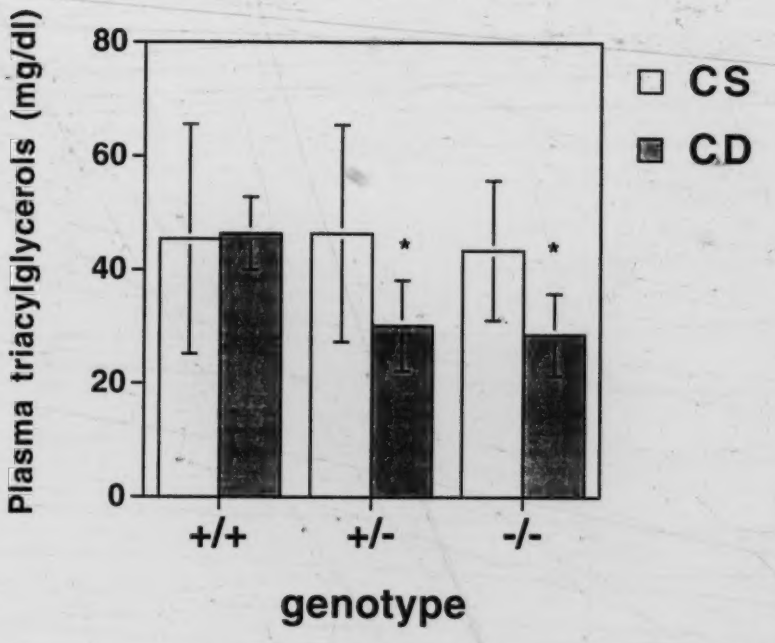


Figure 4.9 **Plasma triacylglycerol levels (measured as glycerol) in mice fed choline-deficient and choline-supplemented diets.**

Blood was collected from mice by cardiac puncture, and plasma isolated by low-speed centrifugation. The level of triacylglycerol in the plasma was measured using a GPO-Trinder kit (Sigma), following the manufacturer's instructions, but adapted for microtiter plate analysis. *P< 0.05 compared to (+/+). *(Performed by L. Yu)*

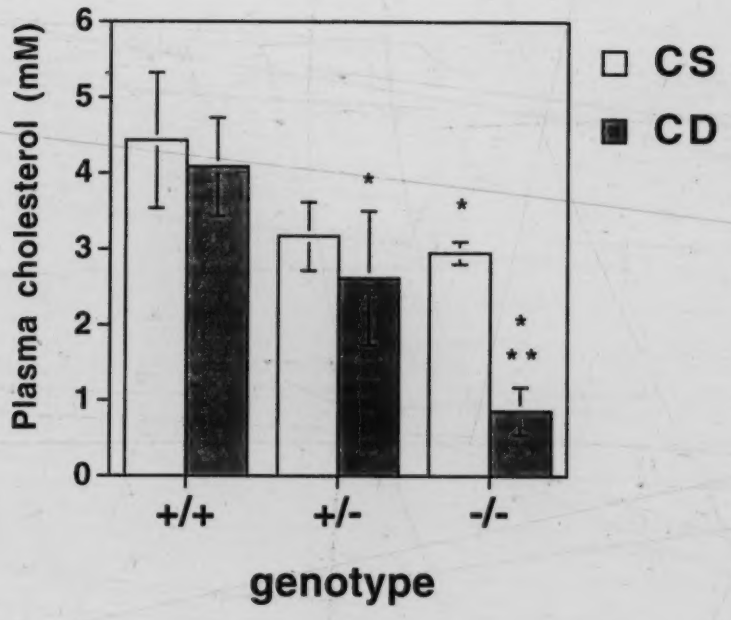


Figure 4.10 **Plasma total cholesterol levels in mice fed choline-deficient and choline-supplemented diets.**

Cholesterol in plasma was measured using a Cholesterol 100 kit (Sigma), following the manufacturer's instructions, but adapted for microtiter plate analysis. *P< 0.05 compared to (+/+); **P< 0.05 compared to (+/-). *(performed by L. Yu)*

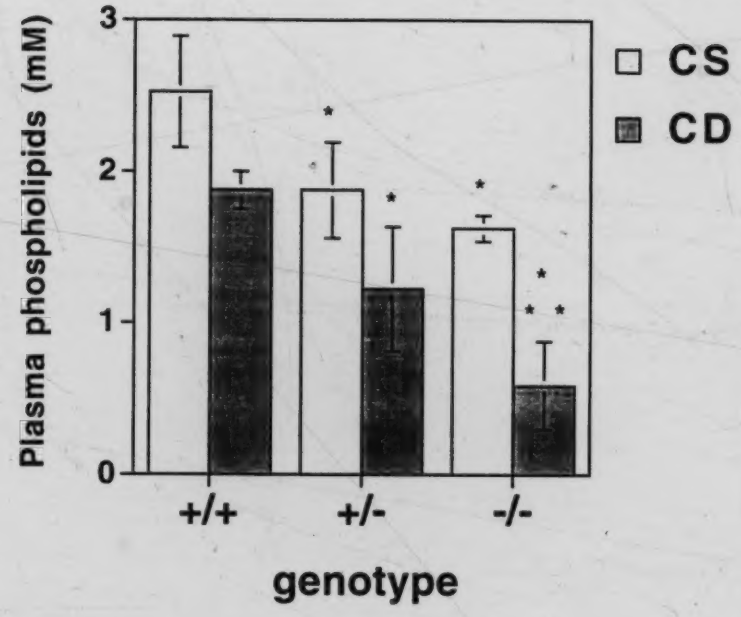
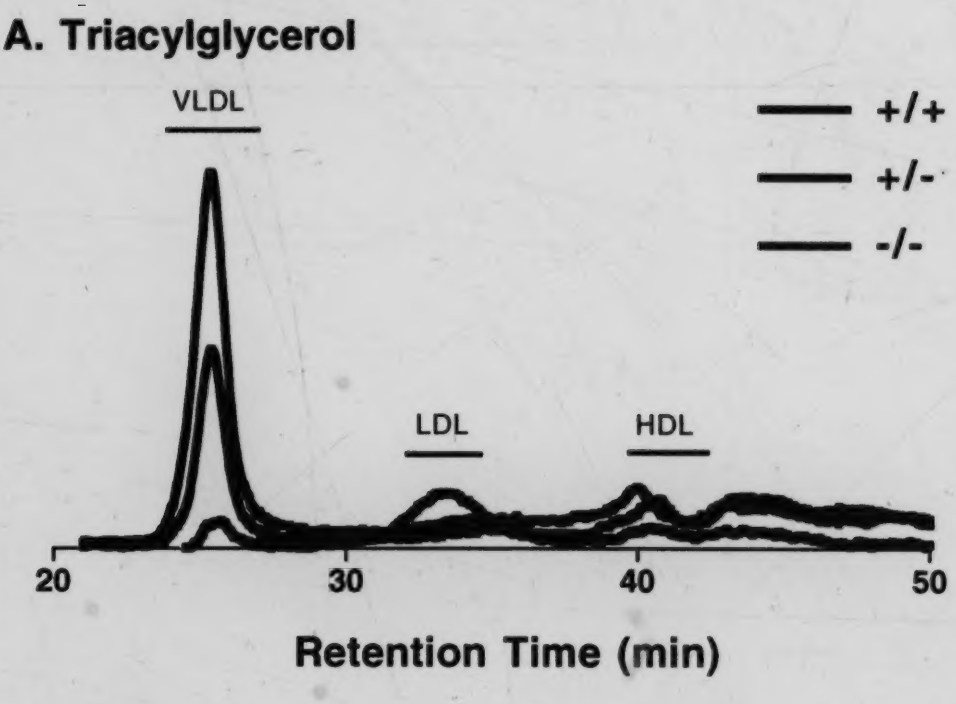


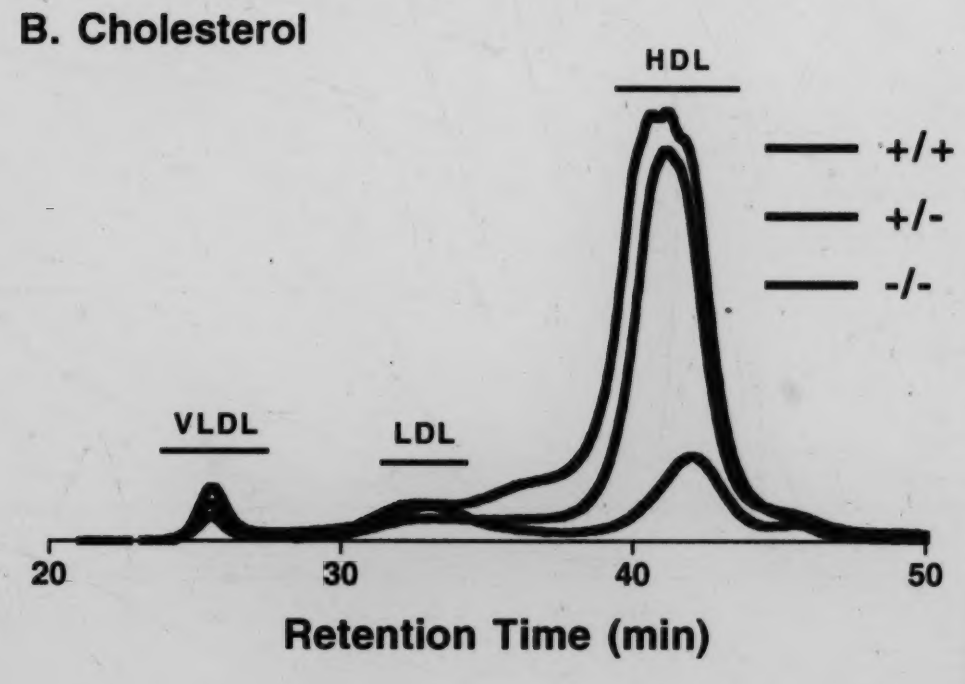
Figure 4.11 **Plasma phospholipid levels in mice fed choline-deficient and choline-supplemented diets.**

Total phospholipids in plasma were measured as for liver (Fig. 4.5A), following extraction of lipids. **P*< 0.05 compared to (+/+); ***P*< 0.05 compared to (+/-).

Figure 4.12 **Plasma lipoprotein profile from mice fed a choline-deficient diet.**

Plasma from mice fed a CD diet was pooled according to genotype, and lipoproteins separated by high-performance liquid chromatography on a System Gold apparatus (Beckman), with a Superose 6 gel filtration column (Pharmacia). (A) An in-line assay for triacylglycerol, which detects glycerol liberated following lipase digestion, using the GPO-Trinder kit (Sigma), following the manufacturer's instructions, was performed. Peaks were identified by comparison to previous runs with lipoprotein standards. (B) An in-line assay for cholesterol, using the Cholesterol 100 kit (Sigma), following the manufacturer's instructions, was performed. Peaks were identified by comparison to previous runs with lipoprotein standards. *(Performed by R. Waite)*


A. Triacylglycerol
VLDL
LDL
HDL
+/+
+/-
-/-
20
30
40
50
Retention Time (min)


B. Cholesterol
HDL
VLDL
LDL
+/+
+/-
-/-
20
30
40
50
Retention Time (min)

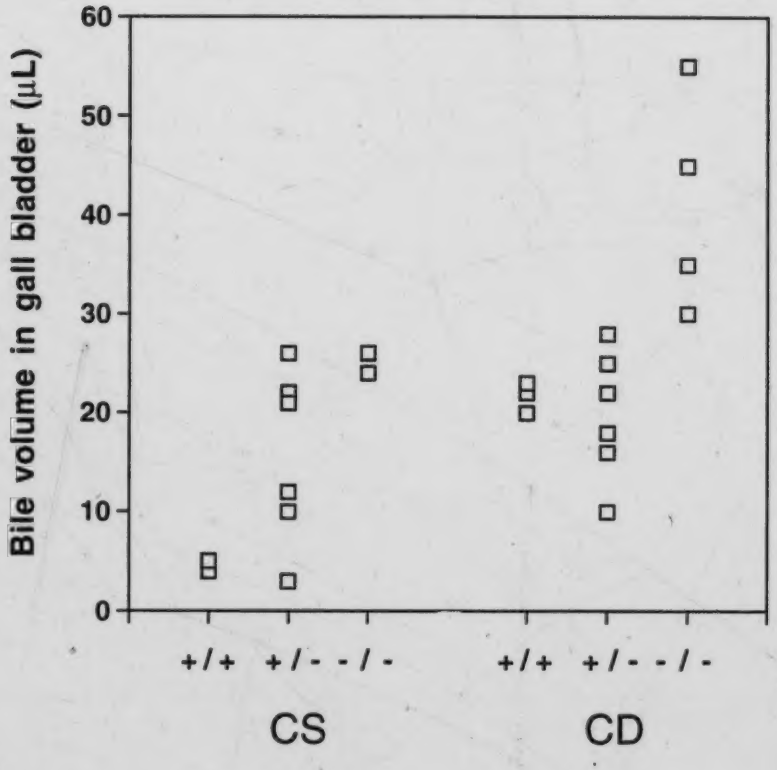


Figure 4.13 **Bile accumulates in the gall bladder of *Pempt* (-/-) mice fed a choline-deficient diet.**

Bile was extracted directly from the gall bladders of mice fed a CS or CD diet for three days, then fasted overnight, and the volume of liquid measured. Values from individual mice are shown. *(Performed by L. Yu)*

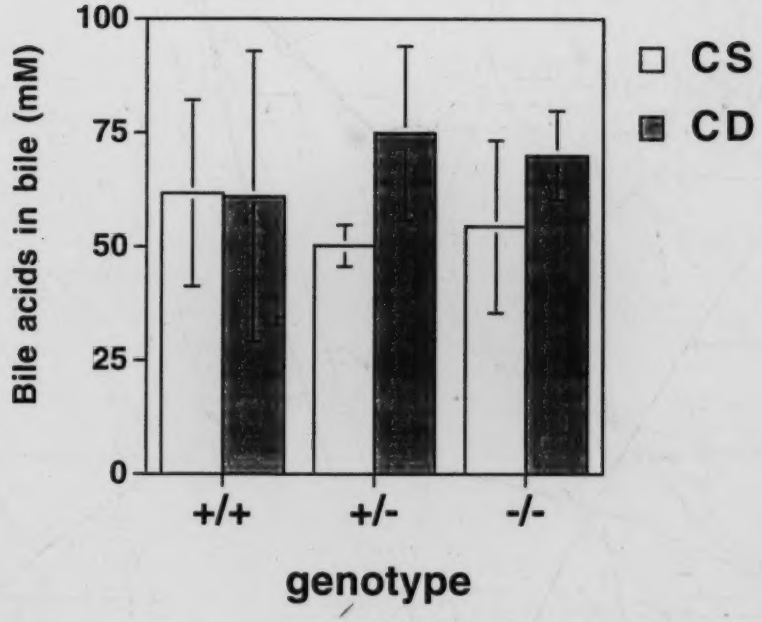


Figure 4.14 **Bile acid concentration in the bile of mice fed choline-deficient and choline-supplemented diets.**

Total bile acids in the bile extracted from the gall bladders were assayed using a Bile Acids kit (Sigma), following the manufacturer's instructions. *(Performed by L. Yu)*

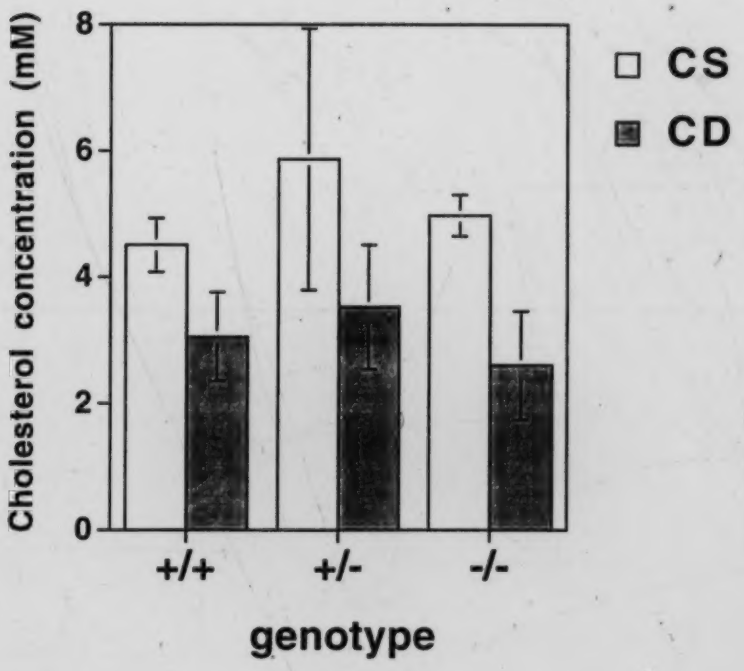
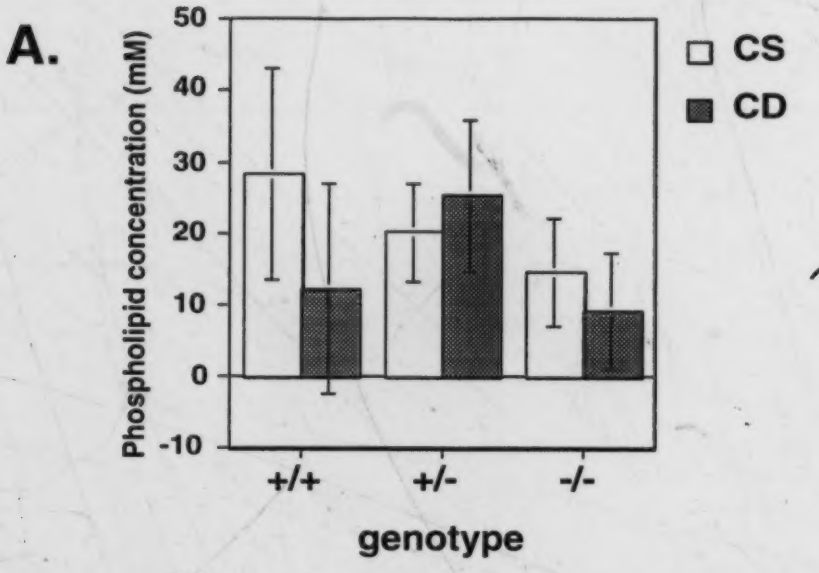


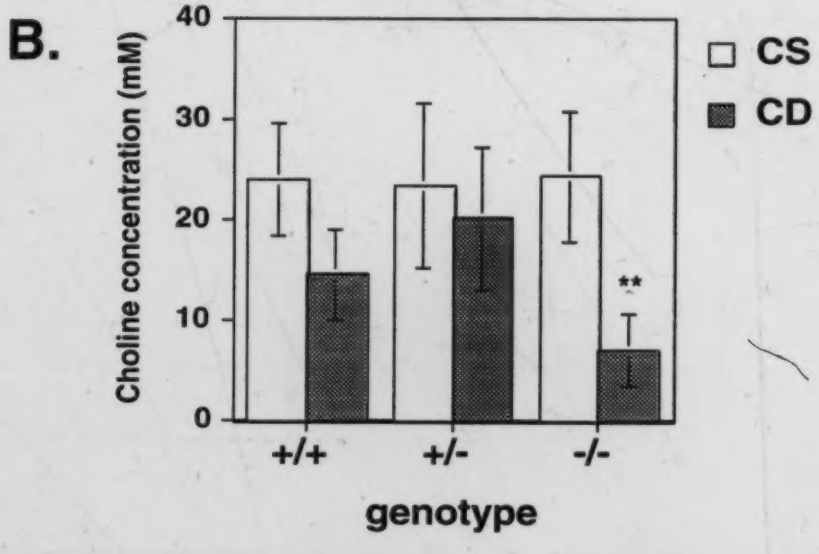
Figure 4.15 **Cholesterol concentration in the bile of mice fed choline-deficient and choline-supplemented diets.**

Total cholesterol in the bile extracted from the gall bladders was diluted 20-fold, and assayed using the Cholesterol 100 kit (Sigma), following the manufacturer's instructions. *(Performed by L. Yu)*

Figure 4.16 **PC concentration in the bile of *Pempt* (-/-) mice fed choline-deficient was markedly reduced.**

(A) Phospholipid in the bile extracted from the gall bladders was assayed by the method of Bartlett (14), without a prior extraction of the lipids. Large sample-to-sample variation was observed, rendering any analysis meaningless. (B) Total choline in the bile extracted from the gall bladders was measured using the Phospholipids B kit (Wako), following the manufacturer's instructions. (C) Lipids were extracted from 4μL of bile pooled according to diet and genotype, and separated by thin-layer chromatography with chloroform/methanol/acetic acid/water (50/15/8/3) as a solvent. PC was visualized by charring with sulfuric acid. $^{**}P < 0.05$ compared to (+/-).*(Performed by R. Waite)*


A.
Phospholipid concentration (mM)
50
40
30
20
10
0
-10
CS
CD
+/+
+/-
-/-
genotype


B.
Choline concentration (mM)
40
30
20
10
0
**
CS
CD
+/+
+/-
-/-
genotype

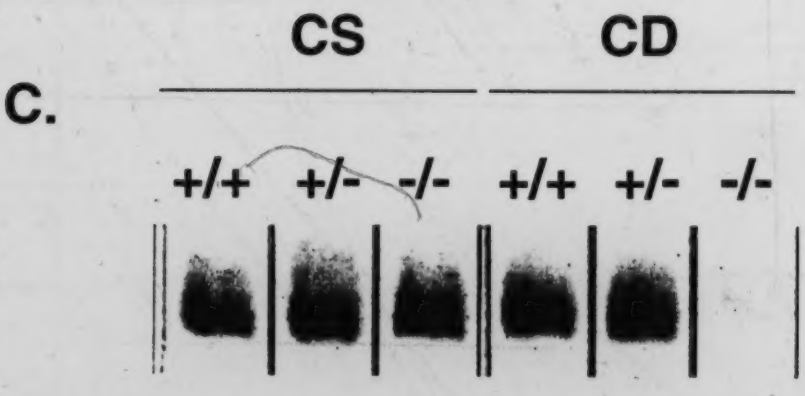
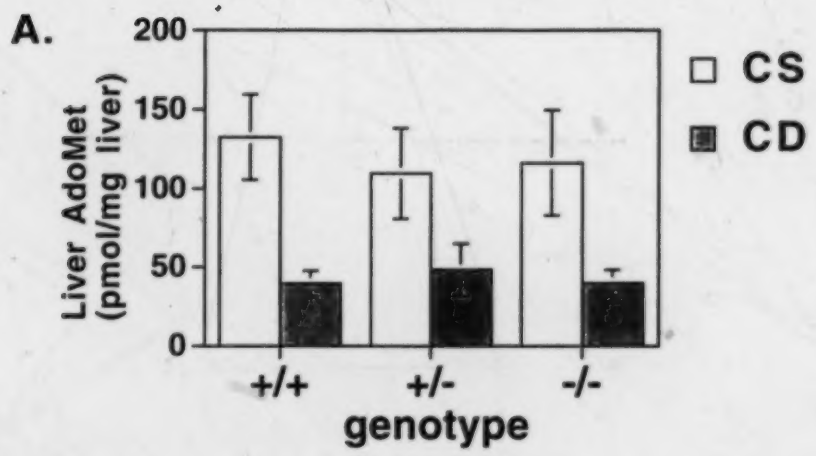
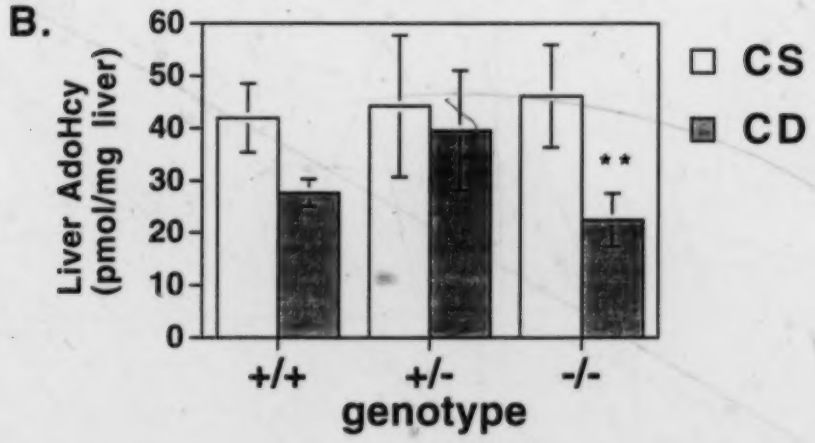
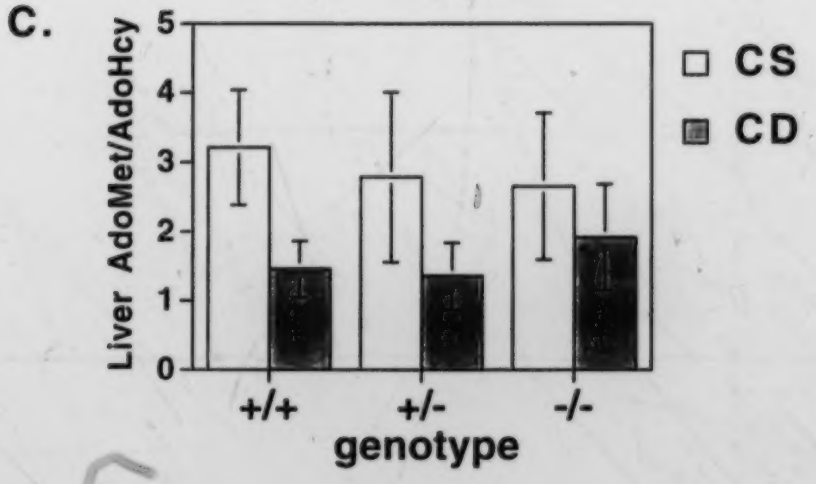

C.
CS
CD
+/+
+/-
-/-
+/+
+/-
-/-

Figure 4.17 **AdoMet and AdoHcy in the livers of mice fed choline-deficient and choline-supplemented diets.**

(A) Hepatic AdoMet levels were measured in liver samples following three days on CD or CS diets, according to the method of Molloy (17). (B) Hepatic AdoHcy levels were measured in liver samples. (C) The AdoMet/AdoHcy ratio was calculated from data above. The ratio was lower in CD mice compared to CS mice. No significant effect of *Pempt* genotype was observed. $^{**}P < 0.05$ compared to (+/-).*(Performed by M.-H. Mar and S. Zeisel)*


A.
200
150
100
50
0
Liver AdoMet
(pmol/mg liver)
+/+
+/-
-/-
genotype
CS
CD


B.
60
50
40
30
20
10
0
Liver AdoHcy
(pmol/mg liver)
**
+/+
+/-
-/-
genotype
CS
CD


C.
5
4
3
2
1
0
Liver AdoMet/AdoHcy
+/+
+/-
-/-
genotype
CS
CD

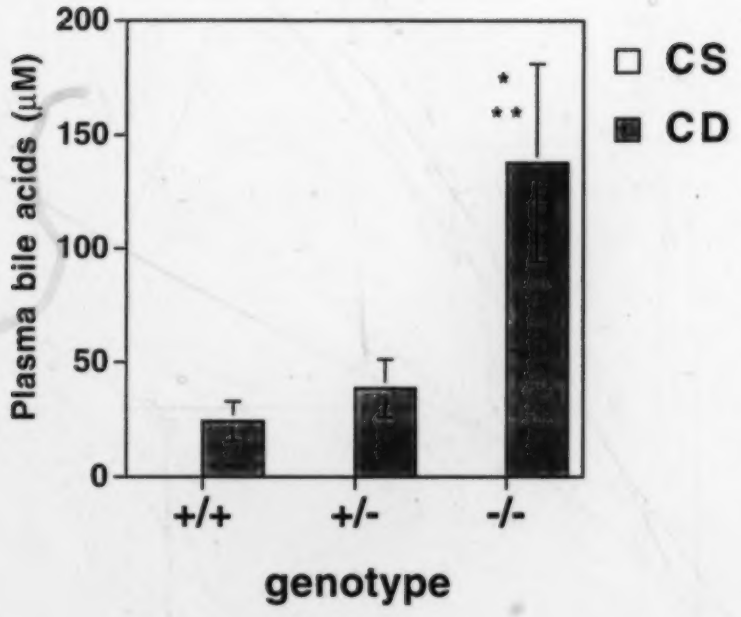


Figure 4.18 **The level of bile acids in the plasma is elevated in *Pempt* (-/-) mice fed a choline-deficient diet.**

Bile acids in the plasma were assayed using a Bile Acids kit (Sigma), and following the manufacturer's instructions, but adapted for microtiter plate analysis. The level of bile acids in all CS mice was less than 1 μM. **P*< 0.05 compared to (+/+); ***P*< 0.05 compared to (+/-). *(Performed by L.Yu)*

diet	CS			CD		
genotype	+/+	+/-	-/-	+/+	+/-	-/-
ALT (IU/L)	ND	ND	ND	33	ND	417
AST (IU/L)	<1	<1	<1	25	33	150

Table 4.1 **Choline-deficiency in *Pempt* (-/-) mice causes aminotransferase leakage into the bloodstream.**

Plasma was pooled according to genotype and diet, and alanine aminotransferase (ALT and aspartate aminotransferase (AST) assayed using the GO & GP Transaminase kit (Sigma), and following the manufacturer's instructions. ND = not detectable. *(Performed by R. Waite)*

References

1. Tinoco, J., Endemann, G., Medwadowski, B., Miljanich, P., and Williams, M. A. (1979) *Lipids* **14**(12), 968-974
2. Hoffman, D. R., Haning, J. A., and Cornatzer, W. E. (1981) *Canadian Journal of Biochemistry* **59**(7), 543-550
3. Ridgway, N. D., Yao, Z., and Vance, D. E. (1989) *Journal of Biological Chemistry* **264**(2), 1203-1207
4. Schneider, W. J., and Vance, D. E. (1978) *European Journal of Biochemistry* **85**(1), 181-187
5. Cui, Z., and Vance, D. E. (1996) *Journal of Biological Chemistry* **271**(5), 2839-2843
6. Zeisel, S. H. (1981) *Annual Review of Nutrition* **1**, 95-121
7. Walkey, C. J., Donohue, L. R., Bronson, R., Agellon, L. B., and Vance, D. E. (1997) *Proceedings of the National Academy of Science, USA* **in press**
8. Meader, R. D., and Williams, W. L. (1957) *American Journal of Anatomy* **100**(2), 168-203
9. Williams, W. L., Cardle, J. B., and Meader, R. D. (1959) *Yale Journal of Biology and Medicine* **31**, 263-270
10. Yoshida, L. S., Miyazawa, T., Fujimoto, K., and Kaneda, T. (1990) *Lipids* **25**(9), 565-569
11. Haines, D. S. M. (1966) *Canadian Journal of Biochemistry* **44**, 45-57
12. Vance, D. E., and Pelech, S. L. (1984) *Trends in Biochemical Science* **9**, 17-20
13. Yao, Z. M., Jamil, H., and Vance, D. E. (1990) *Journal of Biological Chemistry* **265**(8), 4326-4331
14. Kuksis, A., and Mookerjea, S. (1978) *Nutrition Reviews* **36**(7), 201-207

15. Yao, Z. M., and Vance, D. E. (1988) *Journal of Biological Chemistry* **263**(6), 2998-3004

16. Mookerjea, S., Park, C. E., and Kuksis, A. (1975) *Lipids* **10,** 374-382

17. Yao, Z. M., and Vance, D. E. (1990) *Biochemistry & Cell Biology* **68**(2), 552-558

18. Hamilton, R. L., Havel, R. J., Kane, J. P., Blaurock, S. E., and Sata, T. (1971) *Science* **172,** 475-478

19. Smit, J. J. M., Schinkel, A. H., Oude Elferink, R. P. J., Groen, A. K., Wagenaar, E., van Deemter, L., Mol, C. A. A. M., Ottenhoff, R., van der Lugt, N. M. T., van Roon, M. A., van der Valk, M. A., Offerhaus, G. J. A., Berns, A. J. M., and Borst, P. (1993) *Cell* **75,** 451-462

20. Mato, J. M., Alvarez, L., Corrales, F. J., and Pajares, M. A. (1994) in *The Liver: Biology and Pathobiology* (Arias, I. M., Boyer, J. L., Fausto, N., Jakoby, J. M., Schachter, D. A., and Shafritz, D. A., eds), 3rd Ed., pp. 461-469, Raven Press Ltd., New York

21. Marshall, W. J. (1995) in *Clinical Chemistry*, pp. 72-87, Mosby, London

22. Zeisel, S. H. (1990) *Journal of Nutritional Biochemistry* **1,** 332-349

23. Esko, J. D., Wermuth, M. M., and Raetz, C. R. (1981) *Journal of Biological Chemistry* **256**(14), 7388-7393

24. Walmsley, R. N., and White, G. H. (1983) *A Guide to Diagnostic Clinical Chemistry*, Blackwell Scientific Publications, Carlton, Australia

25. Glickman, R. M., and Sabesin, S. M. (1994) in *The Liver: Biology and Pathobiology* (Arias, I. M., Boyer, J. L., Fausto, N., Jakoby, W. B., Schacter, D. A., and Shafritz, D. A., eds), 3rd Ed. Ed., pp. 391-414, Raven Press, Ltd., New York

26. Snyder, F., and Stephens, N. (1959) *Biochimica et Biophysica Acta* **34,** 244-245

Chapter 5

cDNA cloning and chromosomal localization of human PEMT2

5.1 Introduction

Phosphatidylcholine, the most abundant phospholipid in eukaryotic cells, can be synthesized by two major pathways in the liver. The first, called the CDP-choline pathway, converts choline to PC in all mammalian cells. The second, called the PE methylation pathway, occurs only in the mammalian liver. This pathway, which consists of three sequential methylations of phosphatidylethanolamine by *S*-adenosylmethionine (AdoMet), is catalyzed by the enzyme phosphatidylethanolamine *N*-methyltransferase (PEMT).

PEMT has been purified from rat liver (1), and a cDNA cloned from the same tissue (2). Immunolocalization experiments suggest two isoforms of PEMT: PEMT1, which constitutes the majority of PEMT activity, is localized to the endoplasmic reticulum, and PEMT2, corresponding to the cloned cDNA, is localized to the mitochondria-associated membrane (3). The nature of the difference between these two isoforms is unknown, although both are transcribed from the same gene, *Pempt* (4,5).

This thesis addresses the question of why the liver has retained PEMT as an additional source of PC. Mice lacking PEMT activity do not display any difference from their normal counterparts, as long as dietary choline is present (Chapter 3) (5). However, the absence of dietary choline is deleterious to mice lacking PEMT, although normal mice survive relatively unaffected (Chapter 4). Therefore, PEMT is required to maintain hepatic PC biosynthesis when the CDP-choline pathway is insufficient.

A second function for PEMT has been revealed by studying the correlation between hepatocyte growth rates and PEMT expression. During embryogenesis in rats, while hepatocytes are undifferentiated and proliferating rapidly, PEMT expression is low (6,7). At birth, as hepatocyte proliferation slows and final differentiation begins, PEMT expression increases dramatically. When hepatocyte proliferation is induced in adult rats by partial hepatectomy, PEMT expression is inhibited (8). As well, PEMT expression is repressed when non-neoplastic liver growth is induced by lead nitrate (9). In these instances, PEMT2 mRNA levels are reduced, suggesting that the inhibition is occurring at the level of *Pempt* gene expression.

These results demonstrate a correlation between PEMT and the growth state (or differentiation) of hepatocytes. Proliferating, undifferentiated hepatocytes do not express PEMT, but non-proliferating, fully differentiated hepatocytes do. The role of PEMT in actually regulating hepatocyte growth has been investigated. When PEMT2 cDNA is stably transfected into a rat hepatoma cell line (McArdle RH7777), the division time of these cells is increased (10). The repression of cell growth is specific for the expression of PEMT2, as opposed to the transfection process itself. Inhibition of PEMT with 3-deazaadenosine restores normal growth rates, suggesting that it is the methyltransferase activity itself that retards cell division. When tumorigenesis is chemically induced in rat livers, PEMT expression is reduced 60 to 90% very early, at the foci stage (L. Tessitore, unpublished results). Therefore, these results raise the possibility that PEMT could be a candidate for a tumour suppressor.

We are interested in determining whether the apparent growth regulation function is relevant to human liver cancer, the cause of death of up to a million people per year. Although the tumour suppressor p53 is deleted in some hepatocellular carcinomas, not all liver cancers can be explained by the loss of this molecule (11). Possibly PEMT, specifically PEMT2, is eliminated in many human liver cancers. If so, this would further suggest a role for PEMT2 in tumourigenesis. The initial steps in testing this hypothesis are outlined in this chapter.

5.2 Materials and Methods

5.2.1 Screening a human liver cDNA library

A commercially-manufactured cDNA library prepared from the liver of a 40-year-old Caucasian male (Clontech) (gift of Dr. T. Kodama) was screened for human PEMT2 (hPEMT2) clones, using radiolabeled rat PEMT2 (rPEMT2) cDNA as a probe. Approximately 20 million λDR2 phage clones were initially double screened, according to the manufacturers instructions. Putative positive clones were isolated and re-screened. Four rounds of screening were required to fully isolate 3 independent clones, named rp1a-1a, rp3c-1a and rp18b-2a. λ DNA in the clones was converted to plasmid DNA, then isolated using the Wizard Mini-prep kit (Promega), and sequenced. All three clones were completely sequenced in both directions on an Applied Biosystems Inc./Perkin-Elmer model 373A automated sequencer, using primers based on the sequence of the adjacent vector, as well as primers based on the sequence of rPEMT2 cDNA.

5.2.2 Chromosomal mapping of the human PEMT2 gene

Primers based on the 3' untranslated region of the human PEMT2 cDNA (forward: 5'-CTGCAACAGCTTTGCTGAAG-3'; reverse: 5'-GCTCAATGGCCATATGTCG-3') were used to screen the NIGMS v.2 human/rodent somatic cell hybrid panel at the Canadian Genome Analysis and Technology centre under the direction of Dr. S. Scherer. Polymerase chain reactions (PCR) were carried out with an annealing temperature of 55°C in 1.5 mM Mg^{2+}. As well, these primers were used to screen the Genebridge 4 radiation hybrid panel at the same facility, also by PCR (12).

Independently, a second set of primers also based on the 3' untranslated region of the human PEMT2 cDNA was used to screen by PCR the CEPH1 Yeast Artificial Chromosome (YAC) library (forward: 5'-TGAGCTGCAACAGCTTTGCTGAAG-3'; reverse: 5'-GGAATGTGTGGGTTGGAGCTCAATG-3'). This library has a depth of 7 genomes and an average insert size of 430 kb. The screening was carried out by Dr. H. Aburatani at the University of Tokyo.

5.3 Results

5.3.1 Cloning of a human cDNA for PEMT2

An adult human liver cDNA library was screened by hybridization using rat liver PEMT2 cDNA as a probe. From an initial screen of 20 x 10^6 λ phage particles, three clones were isolated that gave strong hybridization signals with the probe. The phage DNA was converted to recombinant plasmid, then

isolated and sequenced. Sequence data revealed a 199 amino acid open reading frame (same as for rat PEMT2), and a polyadenylation signal (AATAAA) (Fig. 5.1). Within this open reading frame, human and rat PEMT2 were 82.3% identical at the nucleotide level. Interestingly, from a point 15 nucleotides upstream of the putative translation initiation codon to the 5' terminus of each insert, there was no identity between the three cDNA clones (Fig. 5.2). As well, none of these upstream regions bore any resemblance to the 5' untranslated region of the rat PEMT2 cDNA. This point of divergence (-15) represents a splice site in the mouse *Pempt* gene (4), therefore, the presence of different 5' upstream regions in the three clones may be a consequence of alternative splicing of the human gene. All three clones contained in-frame stop codons upstream of the translation initiation site.

The 199 amino acid open reading frame of the hPEMT2 clones predicted a protein with a molecular mass of 22.2 kDa and an isoelectric point of 9.6 (for rPEMT2, these values are 22.3 kDa and 8.3). Comparison of the putative human PEMT2 protein sequence to that from rat revealed 80.4% identity and 90.5% similarity (Fig. 5.3). A Kyte-Doolittle analysis of the hydrophobicity of the calculated protein revealed four significant stretches of hydrophobicity, most likely corresponding to transmembrane segments (Fig. 5.4) (13). This hydrophobicity plot closely resembled that for rPEMT2. These results strongly suggest that we have cloned the human version of PEMT2.

Recently, mass cDNA sequencing efforts have been undertaken in order to generate Expressed Sequence Tags (ESTs) that identify every expressed gene in a particular human tissue (14,15). We have scanned the database of ESTs (dbEST) using the BLAST algorithm in order to identify those

corresponding to hPEMT2 (16). Forty-nine human ESTs were identified with significant homology to hPEMT2, representing 33 different cDNAs. Occasional nucleotide differences were observed between the sequence of our clones and those of the ESTs. It is impossible to know at this point whether these differences were due to actual differences between the cDNAs, or due to sequencing errors. The following tissues were represented by these ESTs: total fetus (22 weeks), ovary tumor, pregnant uterus, testis, tonsils, Wilms tumor, fetal liver spleen (22 weeks), fetal lung (22 weeks), placenta, bulk tumors, prostate and pancreatic tumor. Interestingly, the tissue from which our cDNAs were cloned, adult liver, is not on this list. None of these tissues, with the exception of testis, has ever been tested for the expression of PEMT2. In rats, testis does not appear to express the PEMT2 mRNA (2). The EST database was also screened with the sequences for the three different 5'-untranslated regions. The 5'-untranslated region sequence from clone rp1a-1a appeared only in ESTs from fetal liver spleen, while that from clone rp3c-1a did not appear in any ESTs. The 5'-untranslated region sequence from rp18b-2a was found in ESTs from total fetus, pregnant uterus, tonsils, fetal liver spleen, fetal lung, placenta and prostate. In all cases, the 5'-untranslated regions were contiguous with the PEMT2 coding region. Many cDNAs identified by searching dbEST with the coding region of PEMT2 were not also identified by searching dbEST with the 5'-untranslated regions, suggesting that they are either not full-length, or have yet to be sequenced in both directions.

5.3.2 Chromosomal localization of the human PEMT2 gene

The mouse *Pempt* gene localizes to chromosome 11, approximately 31 centimorgans (cM) from the centromere (4). This locus falls on the boundary of

two regions of synteny to human chromosomes: 5q and 17p. Two techniques were used to physically map the human PEMT2 gene to a chromosomal locus: hybrid panel screening and YAC screening. Using primers specific for the 3' untranslated region of the human, but not rat, PEMT2 cDNAs, a somatic cell hybrid panel was screened. Only hybrids containing human chromosome 17 were positive (i. e. the PCR reaction amplified a 178 bp fragment), thus placing the human PEMT gene on this chromosome. Radiation hybrid panel screening to further refine the chromosomal localization was unsuccessful, probably because the PCR conditions were not ideal.

The chromosomal location of the human PEMT2 was also determined by screening YACs, again by PCR using primers based on the 3' untranslated region of the PEMT2 cDNA. Two YACs were positive: 672_C_9 and 943_G_12. These two YACs were searched for other sequences that have previously been mapped, notably sequence tagged sites (STSs), contained in them in databases at the Whitehead Institute and Baylor University . Unfortunately, these two YACs have not been well characterized: no STSs have been reliably attributed to either 672_C_9 or 943_G_12.

Recently, an international effort was undertaken to convert ESTs into STSs, and map them to chromosomal localizations (17). ESTs identified as PEMT2 above were mapped as part of this effort. Radiation hybrid panel mapping using the Genebridge 4 panel, but different PCR primers than above, placed an STS (called sTSG2083) corresponding to the human PEMT2 cDNA within a 4.2 centimorgan (cM) region of chromosome 17, on the p arm and within cytogenetic band 11.2 (Fig. 5.5).

5.4 Discussion

This chapter describes the cloning and characterization of three cDNAs encoding PEMT2 from an adult human liver library. The putative protein encoded by these cDNAs is 80% identical and 90% similar to the rat PEMT2 protein. As well, the patterns of hydropathy of rat and human PEMT2 are virtually identical. Finally, rat and human PEMT2 protein have similar molecular masses and isoelectric points, and an identical number of amino acids. These data strongly support the notion that the cloned cDNAs do in fact encode human PEMT2. The cDNAs have been subcloned into expression vectors and transfected into the rat hepatoma cell line McArdle RH7777. The data from these experiments, not shown in this thesis, further confirm that these cDNAs encode PEMT2.

Experiments on the tissue distribution of PEMT activity in rats have long suggested that PE methylation occurs to a significant degree only in the liver (18). PEMT2 gene expression as well has only been detected in the liver (2). Therefore, when dbEST was scanned for ESTs corresponding to human PEMT2, we were surprised to find non-hepatic tissues such as tonsils and pregnant uterus represented. To our knowledge, none of these tissues have been assayed for PEMT activity in rats, nor have they been tested for PEMT2 gene expression. Whether or not this apparent tissue distribution in human occurs in other organisms as well remains unknown. One of the drawbacks of using ESTs to determine tissue distribution is that it makes no allowances for mRNA abundance. It may be that in the non-hepatic tissues in which PEMT2 ESTs have been detected, the PEMT2 mRNA level is very low, meaning PEMT activity contributes very little to PC biosynthesis. Still, the rare PEMT2 mRNAs

could be converted to cDNAs and sequenced. A multiple tissue RNA blot would be a very useful tool for determining the level of PEMT2 gene expression in non-hepatic tissues.

Another interesting difference between rat and human PEMT2 revealed by ESTs is the expression of PEMT2 in fetal tissues, notably mid-gestation fetal liver and spleen. This result contradicts the situation in rats, where PEMT2 expression is not detectable until birth (7). Again however, the fetal ESTs may represent very rare PEMT2 mRNAs.

The three different cloned cDNAs each contained a different 5'-untranslated region, probably arising from alternative splicing of PEMT2 transcripts. Matching these different regions to PEMT2 ESTs suggested a correlation to tissue distribution: rp1a-1a's 5'-untranslated region was present in ESTs from fetal liver and spleen only, while rp18b-2a's 5'-untranslated region was present in ESTs from a variety of tissues. The 5'-untranslated region from rp3c-1a did not appear in any ESTs. This result leads to the hypothesis of multiple promoters for the human PEMT2 gene, with different promoters for different tissues (Fig. 5.6). However, all three promoters would be at least partly functional in the adult human liver, because all three differentially-spliced versions of the PEMT2 transcript are represented by cDNAs. Alternatively, only one promoter may be present, with the different 5'-untranslated regions generated solely by differential splicing according to tissue.

Using radiation hybrid panels, the human PEMT2 gene has been physically mapped to a 4.2 cM region on 17p11.2. The region of the human genome is syntenic to that for the region surrounding the mouse *Pempt* gene.

Thirteen other genes also map to this interval, including the genes for the sodium/glucose co-transporter and fatty aldehyde dehydrogenase (17). It is interesting to note that a 5 MB deletion of 17p11.2 has been implicated as the cause of Smith-Magenis syndrome (SMS), which results several facial and body abnormalities and mental retardation (19,20). This is an autosomal dominant disorder: patients are hemizygous for the genes in the deleted region. Recently, it has been found that the human PEMT gene is one of the genes deleted in this syndrome (S. Elsea, personal communication). However, it is doubtful that PEMT is the crucial gene for this syndrome. It is unlikely that loss of one copy of a phospholipid biosynthesis gene would result in the phenotype observed. Mice heterozygous for the disrupted *Pempt* gene show no similarity of phenotype with SMS patients (5). As well, the tissues that are known to be affected by the SMS symptoms do not correspond to those known to express PEMT. It is more likely that hemizygosity of another gene closely linked to PEMT is responsible for SMS.

The purpose of cloning and characterizing the human PEMT2 cDNA was as a first step in the characterization of its gene, and the investigation of the role of PEMT as a candidate liver tumour suppressor. Notably, 17p is frequently deleted in primary liver cancer (21). We have recently obtained Bacterial Artificial Chromosome (BAC) clones which will be analyzed for the presence of the human PEMT2 gene. The gene will be characterized, in particular with respect to the alternative splicing hypothesis (Fig. 5.6). As well, the BAC clones will be used as a probe for Fluorescence In-Situ Hybridization (FISH) mapping, to confirm the physical mapping of the PEMT2 gene to chromosome 17p11.2. Finally, the BAC clones will form the basis for the design of a series of PEMT gene-specific PCR primer pairs, to be used for loss of heterozygosity studies in

human hepatocellular carcinomas. In this way, the possible role of PEMT as a regulator of hepatocyte growth in the human liver will be evaluated.

-15 GCAGACTTCTGCGTT**ATG**ACCCGGCTGCTGGGCTACGTGGACCCCCTGG

+35 ATCCCAGCTTTGTGGCTGCCGTCATCACCATCACCTTCAATCCGCTCTA

+85 CTGGAATGTGGTTGCACGATGGGAACACAAGACCCGCAAGCTGAGCAGG

+135 GCCTTCGGATCCCCCTACCTGGCCTGCTACTCTCTAAGCGTCACCATCC

+185 TGCTCCTGAACTTCCTGCGCTCGCACTGCTTCACGCAGGCCATGCTGAG

+235 CCAGCCCAGGATGGAGAGCCTGGACACCCCCGCGGCCTACAGCCTGGGC

+285 CTCGCGCTCCTGGGACTGGGCGTCGTGCTCGTGCTCTCCAGCTTCTTTG

+335 CACTGGGGTTCGCTGGAACTTTCCTAGGTGATTACTTCGGGATCCTCAA

+385 GGAGGCGAGAGTGACCGTGTTCCCCTTCAACATCCTGGACAACCCCATG

+435 TACTGGGGAAGCACAGCCAACTACTTGGGCTGGGCCATCATGCACGCCA

+485 GCCCCACGGGCCTGCTCCTGACGGTGCTGGTGGCCCTCACCTACATAAT

+535 GGCTCTCCTATACGAAGAGCCCTTCACCGCTGAGATCTACCGGCAGAAA

+585 GCCTCCGGGTCCCACAAGAGGAGC**TGA**TTGAGCTGCAACAGCTTTGCTG

+635 AAGGCCTGGCCAGCTTCCTGGCCTGCCCCAAGTGGCAGGCCCTGACGCA

+685 GGGCGAGAATGGTGCCTGCTGCTCAGGGTTCGCCCCCGGCGTGGGCTGC

+735 CCCAGTGCCTTGGAACCTGACTGCCTTGGGGACCCTGGACGTGCCGACA

+785 TATGGCCATTGAGCTCCAACCCACACATTCCCATTCACC<u>AATAAA</u>GGCA

+835 CCCTGACCCCAAAAAAAAAAAAAAAAAAAA

Figure 5.1 **Nucleotide sequence of human liver PEMT2 cDNA.**

The nucleotide sequence of human liver PEMT2 was based on the sequence of clones rp1a-1a, rp3c-1a and rp18b-2a. Numbering is based on +1 as the first nucleotide of the putative translation start codon. The translation start codon (ATG) and the stop codon (TGA) of the largest open reading frame are highlighted in boldface. The polyadenylation signal is underlined.

rp1a-1a

-131 GCGACCATAAAGCCTCTTCCTTACCCATGCTTTGGGGTGTTAACAGCTG

AGGCTATTCGTCGGTGACCTGTGGGACTCGAGCTATTCCTGCAGCTCAG

CAGACCTCCTGGCCGTG -16

rp3c-1a

-78 GGATCCGCGGCGAGGAGAGGCACACCACTATTGTTTTAATTTGCTTTTA

CCTGATTCCTG -16

rp18b-2a

-115 GCGGATGAAGAGATCTGGGAACCCGGGAGCCGAGGTAACGAACAGCTCG

GTGGCAGGGCCTGACTGCTGCGGAGGCCTCGGCAATATTGATTTTAGACA -16

Figure 5.2 **Variation in the 5'-untranslated region of the cDNA clones.**

The 5' end of each of the three isolated cDNA clones (rp1a-1a, rp3c-1a and rp18b-2a) was sequenced. The regions were identified as untranslated based on the largest open reading , as shown in figure 1. In-frame stop codons upstream of the translation initiation codon are underlined.

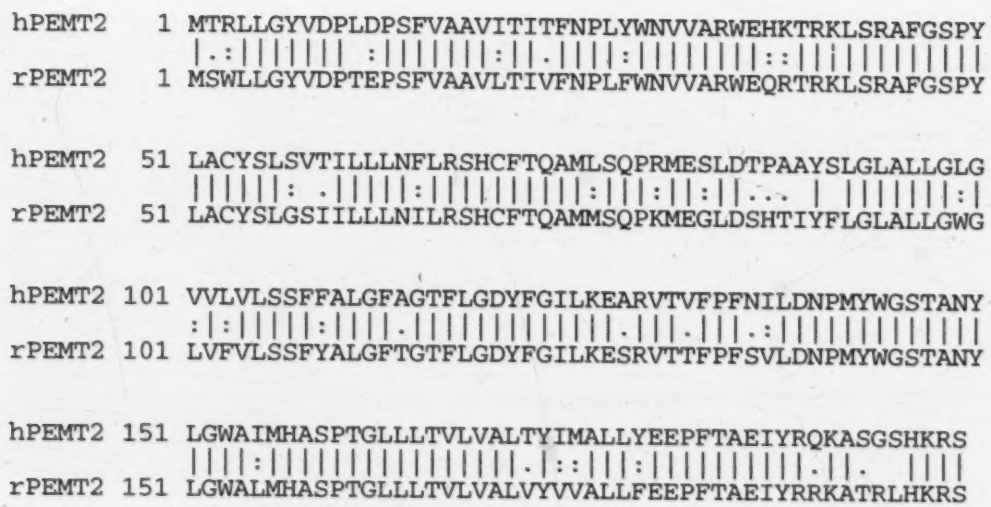


Figure 5.3 **Comparison of human and rat PEMT2 protein sequences.**

The protein sequences of PEMT2 from human (above) and rat (below) liver were determined from the nucleotide sequence of their respective cDNAs and compared using the Gap program of the GCG sequence analysis software. Vertical lines represent identical amino acids, while dots represent conserved amino acid substitutions. Two dots represent strongly similar amino acids, while one dot represents weakly similar amino acids.

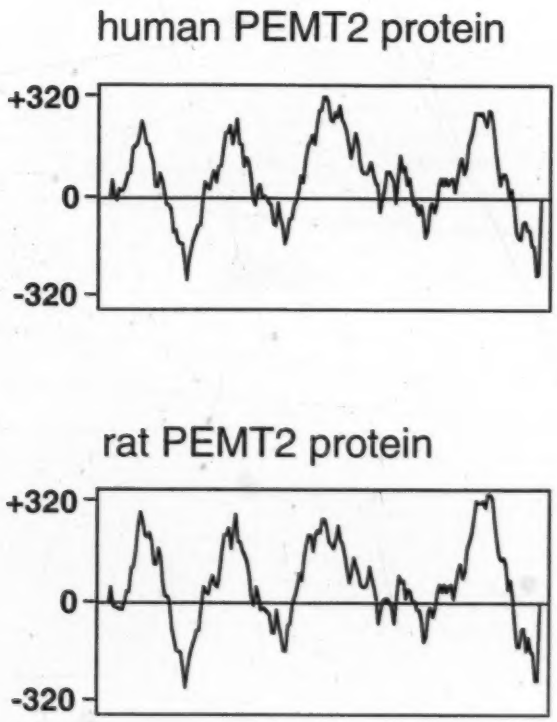


Figure 5.4 **Hydrophobicity analysis of human PEMT2.**

The hydrophobicity profile of human PEMT2 protein was calculated by the method of Kyte and Doolittle (13). The vertical axis indicates hydrophobicity (the higher the value, the greater the hydrophobicity), while the horizontal axis denotes amino acid position.

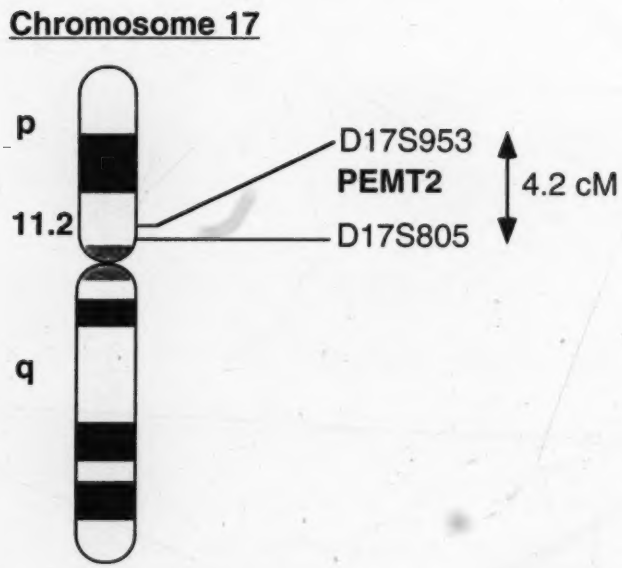


Figure 5.5 **The human PEMT2 gene maps to chromosome 17p11.2.**

A schematic of human chromosome 17 is shown above. The interval which the PEMT2 gene maps, based on radiation hybrid mapping of an STS based on PEMT2 EST sequences. This region is bounded by the STSs D17S953 and D17S805.

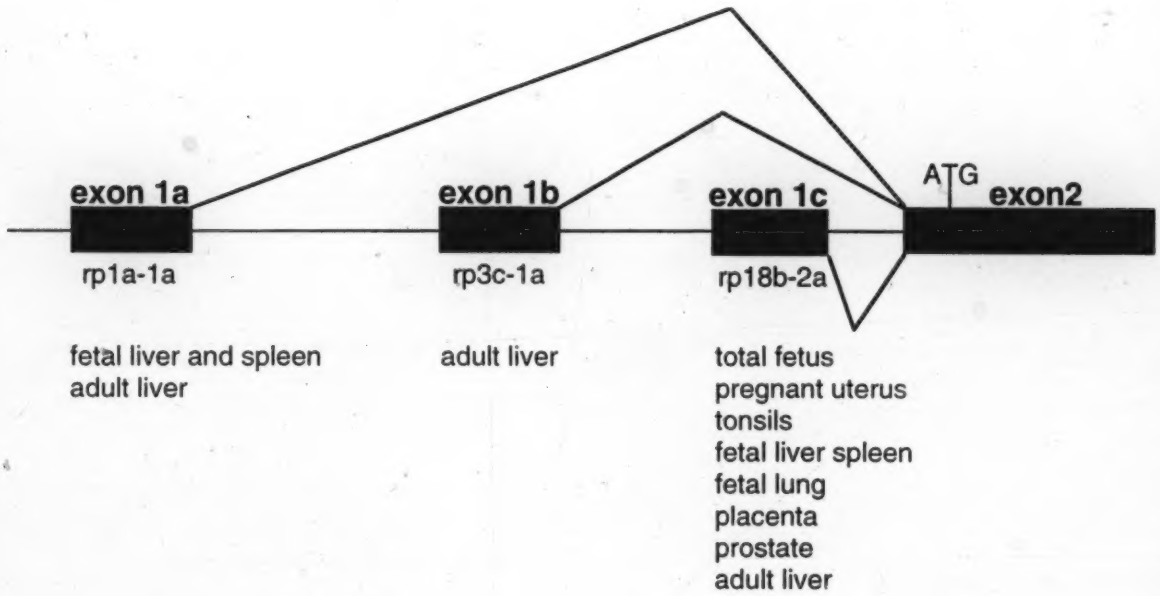


Figure 5.6 **Possible structure of the human PEMT2 gene.**

A diagram of a potential structure of the 5' region of the human PEMT2 gene is shown above. Black boxes represent exons, while the lines joining the three different exon 1s (1a, 1b and 1c) represent alternative splicings. The cDNA clones on which each exon is based in shown below, along with possible tissue distribution based on ESTs and this cloning. The arrangement of exons 1a, 1b and 1c with respect to each other is arbitrary; in reality they could occur in any arrangement. The translation start site (ATG) in exon 2 is also shown.

References

1. Ridgway, N. D., and Vance, D. E. (1987) *Journal of Biological Chemistry* **262**(35), 17231-17239

2. Cui, Z., Vance, J. E., Chen, M. H., Voelker, D. R., and Vance, D. E. (1993) *Journal of Biological Chemistry* **268**(22), 16655-16663

3. Vance, J. E. (1990) *Journal of Biological Chemistry* **265**(13), 7248-7256

4. Walkey, C. J., Cui, Z., Agellon, L. B., and Vance, D. E. (1996) *Journal of Lipid Research* **37**(11), 2341-2350

5. Walkey, C. J., Donohue, L. R., Bronson, R., Agellon, L. B., and Vance, D. E. (1997) *Proceedings of the National Academy of Science, USA* **in press**

6. Pelech, S. L., Power, E., and Vance, D. E. (1983) *Canadian Journal of Biochemistry & Cell Biology* **61**(11), 1147-1152

7. Cui, Z., Shen, Y. J., and Vance, D. E. (1997) *Biochimica et Biophysica Acta* **1346**(1), 10-16

8. Houweling, M., Cui, Z., Tessitore, L., and Vance, D. E. (1997) *Biochimica et Biophysica Acta* **1346**(1), 1-9

9. Tessitore, L., Cui, Z., and Vance, D. E. (1997) *Biochemical Journal* **322**(Pt 1), 151-154

10. Cui, Z., Houweling, M., and Vance, D. E. (1994) *Journal of Biological Chemistry* **269**(40), 24531-24533

11. Buetow, K. H., Sheffield, V. C., Zhu, M., Zhou, T., Shen, F.-M., Hino, O., Smith, M., McMahon, B. J., Lanier, A. P., London, W. T., Redeker, A. G., and Govindarajan, S. (1992) *Proceedings of the National Academy of Science, USA* **89**, 9622-9626

12. Walter, M. A., Spillett, D. J., Thomas, P., Weissenbach, J., and Goodfellow, P. N. (1994) *Nature Genetics* **7**, 22-28

13. Kyte, J., and Doolittle, R. F. (1982) *Journal of Molecular Biology* **157,** 105-132

14. Boguski, M. S. (1995) *Trends in Biochemical Sciences* **20,** 295-296

15. Adams, M. D.*et al.* (1995) *Nature* **377,** Supplement

16. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) *Journal of Molecular Biology* **215,** 403-410

17. Schuler, G. D. *et al.* (1996) *Science* **274,** 540-546

18. Bremer, J., and Greenberg, D. M. (1961) *Biochimica et Biophysisca Acta* **46,** 205-216

19. Smith, A. C. M., McGavran, L., Robinson, J., Waldstein, G., Macfarlane, J., Zonona, J., Reiss, J., Lahr, M., Allen, L., and Magenis, E. (1986) *American Journal of Medical Genetics* **24,** 393-414

20. Stratton, R. F., Dobyns, W. B., Greenberg, F., DeSana, J. B., Moore, C., Fidone, G., Runge, G. H., Feldman, P., Sekhon, G. S., Pauli, R. M., and Ledbetter, D. H. (1986) *American Journal of Medical Genetics* **24,** 421-432

21. Ozturk, M. (1995) in *Molecular Biology and Biochemistry* (Meyers, R. A., ed), pp. 516-518, VCH, New York

Chapter 6

Summary, Conclusions and Future Studies

6.1 Summary and Conclusions

The experiments outlined in this thesis represent an attempt to determine a physiological purpose for PE methylation as a biosynthetic source of PC in the mammalian liver. Fully-differentiated hepatocytes, like all eukaryotic cells, can synthesize PC via the CDP-choline pathway. Therefore, the reason why these cells have retained PE methylation, catalyzed by PEMT and believed to generate between 20 and 40% of hepatic PC (1), is not immediately obvious. However, there are several possibilities, as outlined in Chapter 1. First, PE methylation is widely believed to be the only endogenous source of choline, at least outside the brain. Second, the liver secretes bile and VLDL, both of which require PC, perhaps derived primarily from PE methylation. PEMT has also been hypothesized to be responsible for generating certain molecular species of PC. Finally, recent results suggest that PEMT may play a role in regulating the growth rate of hepatocytes.

One method to investigate the physiological role of PEMT would be to eliminate its activity, and examine the consequences. This is precisely the approach described in this thesis:

Chapter 2 describes the cloning and characterization of the mouse gene encoding PEMT2, named *Pempt2*. Analysis of λ and P1 clones revealed the PEMT2 gene to span at least 35 kb, divided into 7 exons and 6 introns. Two transcription start sites were revealed, 139 and 148 base pairs upstream of the translation start site. Interspecific backcross mapping placed *Pempt2* on mouse chromosome 11, approximately 31 centimorgans from the centromere.

Knowledge of the structure of *Pempt2* allowed the design and construction of a vector for the disruption of this gene by homologous recombination in embryonic stem cells. Such a vector, where part of exon 2 containing the translation start site was replaced by a neomycin-resistance cassette, was constructed and transfected into embryonic stem cells, which were then screened for homologous recombination. Those ES cell clones where homologous recombination took place were microinjected into mouse blastocysts to generate chimeric mice. These mice were bred to generate three genotypes of mice: *Pempt2*(+/+), where both copies of *Pempt2* are intact; *Pempt2*(+/-), where one copy of *Pempt2* is intact and one copy is disrupted; and *Pempt2*(-/-), where both copies of *Pempt2* are disrupted. Chapter 3 describes the consequences of this gene disruption. *Pempt2*(-/-) mice contained virtually no PEMT activity in homogenates from their livers, suggesting that all PEMT activity is encoded by *Pempt2* (hence the subsequent renaming of the gene to *Pempt*). However, phospholipid levels in the mice lacking PEMT are minimally affected. Activity in the membrane-bound form of CT, which catalyzes the rate-limiting step of the CDP-choline pathway, was increased 60%, suggesting a stimulation of this pathway to compensate for the loss of PEMT and maintain hepatic PC levels. In *Pempt2*(+/+) and (+/-), radiolabeled methionine, the precursor to AdoMet which is the methyl group donor for PEMT, is initially incorporated into PC in the liver, but after time is found throughout the body, suggesting that choline formed by PE methylation can be exported to other organs. In *Pempt2* (-/-) mice, radiolabeled methionine is not incorporated into PC in any tissue to a significant extent, confirming *in vivo* the elimination of PEMT activity. The *Pempt2* (-/-) displayed no obvious behavioural, pathophysiological or morphological defects. These results suggest that mice are able to compensate for the loss of PEMT without any apparent deleterious

effects, at least as long as they are fed a standard laboratory chow diet containing choline.

In chapter 4, the importance of PEMT as an endogenous source of choline was examined by feeding *Pempt* (-/-) mice a diet completely lacking choline. In this way, the net biosynthesis of PC is inhibited. The combination of choline and PEMT deficiency is lethal: the mice lose weight, and develop oversized fatty livers. Elevated levels of transaminases and bile in the plasma (which is yellow, indicating jaundice) suggest massive liver damage. PC falls to 37% of total hepatic phospholipid from >50% when either dietary choline or PEMT is present. VLDL is eliminated from the plasma, and replaced, at least in part, with an LDL-sized particle. HDL is also decreased. The level of phospholipid (primarily PC) in the plasma is reduced. Bile accumulates in the gall bladder, but with a significantly lower concentration of PC. This result points to a physiological role for PEMT: the biosynthesis of PC and choline in the absence of dietary choline. Inhibition of PC biosynthesis in this animal model is lethal, demonstrating the importance of this molecule in eukaryotic life.

Chapter 5 describes initial steps in the examination of the role of PEMT in human hepatocellular carcinoma. The inhibition of the growth of hepatoma cell lines by transfection with PEMT2 cDNA suggests that this enzyme may have a role as a tumour suppressor. In order to examine the role PEMT2 as a tumour suppressor at a genetic level, three cDNA clones for human PEMT2 were isolated from an adult liver library and characterized. Sequencing revealed that the protein encoded by the clones was 80% identical and 90% similar to rat PEMT2 at the amino acid level, with a similar number of amino acids, molecular mass, hydropathy plot and isoelectric point. Analysis of ESTs homologous to

the clones suggested expression of PEMT2 in several tissues not yet examined for PEMT activity. Interestingly, the three clones had different 5'-untranslated regions, suggesting alternative splicing, perhaps according to tissue. The human PEMT2 gene was mapped to chromosome 17p11.2 by somatic cell and radiation hybrid mapping. This location, syntenic to that of mouse *Pempt* , is frequently deleted in primary human liver cancers.

6.2 Future studies

6.2.1 Remnant PE methylation activity

A close look at figure 3.2 reveals that in *Pempt2* (-/-) mice, there remains a low level of PE methylation activity (approximately 20% that in normal mice) with exogenous PE as a substrate, but not PMME or PDME. This result raises the possibility of a second PE methylation activity responsible only for the conversion of PE to PMME. Such a situation would mirror that in yeast and other lower eukaryotes, which have two PEMT activities: one (*CHO2/PEM1*) catalyzing the first methylation and one (*OPI3/PEM2)* catalyzing all three methylations (2-6). Initial studies suggest that PMME may accumulate in the livers of *Pempt* (-/-) mice. However, no radiolabeled PMME accumulates when [L-*methyl*-^{3}H]methionine is injected into the bloodstream, implying a very fast catabolism of PMME. It is worth noting that non-enzymatic methylation by AdoMet has been observed, particularly under the basic conditions of our assay (7). Therefore, in order to determine whether the remaining PE methylation activity is enzymatic or chemical, homogenates from *Pempt* (-/-) mice could be heated to eliminate enzyme activity, then assayed. If there is no difference in activity between heated and non-heated samples, chemical rather than

enzymatic PE methylation is the most likely explanation for the remaining activity. If the remaining activity is enzymatic, assay conditions could be varied to determine optimum conditions. In particular, conditions matching the optimum for yeast *CHO2/PEM1* could be attempted (3,4). Eventually, this second PE methylating enzyme could be purified, cloned and "knocked out" in mice.

6.2.2 PEMT at weaning

Histological examination of livers from mice fed a CS diet for three days immediately following weaning revealed extensive vacuolization in samples from *Pempt* (-/-), but not in samples from *Pempt* (+/-) and *Pempt* (+/+) mice. This phenotype disappeared within 5 weeks. One possible explanation is that PEMT is important for the normal metabolism of mother's milk, but not a standard laboratory chow diet. Specifically, the phenotype may be due to choline-deficiency in the milk, but this is unlikely, since neonatal animals and humans have higher blood choline concentrations than adults, and rat milk is a rich source of choline (8,9). Still, the infant mice may require PEMT to fulfill PC and choline requirements. Therefore, experiments with *Pempt* (-/-) mice during post-natal development should be performed, examining fat accumulation in the liver and hepatic PC levels.

6.2.3 The role of PEMT in lipoprotein secretion

Previous experiments on isolated rat hepatocytes with inhibitors suggest that PEMT activity is not strictly required for lipoprotein secretion (10,11). However, inhibitors may not completely block PE methylation, and may have non-specific effects, thus complicating the analysis (12). The lack of PEMT in

Pempt (-/-) means that the role of this enzyme in lipoprotein secretion could finally be settled. As shown in Chapter 4, the combination of PEMT- and choline-deficiency greatly alters circulating lipoprotein metabolism. By isolating hepatocytes, secretion of lipoproteins, rather than 'steady-state' lipoprotein levels, can be measured. As mentioned above, this technique has previously been used to study lipoprotein secretion in rat hepatocytes, therefore, the protocol should be easily adaptable to mice. The fate of radioactive precursors such oleate and glycerol added to hepatocyte cultures would give an indication of the role of PC biosynthesis in lipoprotein secretion. Experiments could be performed under both choline-supplemented and choline-deficient conditions. As well, the nature of the LDL-sized particle containing triacylglycerol in the plasma of *Pempt* (-/-) mice fed a CD diet (Fig. 4.12) could be ascertained by testing for the presence of apolipoprotein B, and electron microscopy. Finally, feeding mice a high fat diet (20 - 40% fat) will challenge the capacity of *Pempt* (-/-) for lipoprotein metabolism, perhaps revealing a role for PEMT in this process that is not evident under standard laboratory chow diet (9% fat).

6.2.4 The role of PEMT in generating unsaturated PC

It has long been hypothesized that PEMT is responsible for generating unsaturated species of PC, due to the highly unsaturated nature of PE (13). However, at least in isolated rat hepatocytes, this does not appear to be the case (14). Now that whole animals lacking PEMT have been constructed, this hypothesis can be more extensively tested. The fatty acid composition of hepatic PC can be determined by gas chromatography from *Pempt* (+/+), (+/-) and (-/-) mice. As well, the fatty acid composition of bile and plasma lipoprotein PC from these same mice could be tested. Any differences observed would

suggest a role for PEMT in determining the fatty acid composition of PC from any of these tissues.

6.2.5 The influence of PEMT on choline metabolism

In all tissues, choline serves as a precursor of PC. However, in some tissues, choline has other metabolic fates (Fig. 1.4). In the brain, choline is converted to acetylcholine, a neurotransmitter. In the liver and kidney, choline is irreversibly oxidized to betaine, which contributes methyl groups to the one-carbon pool. Eventually, methyl groups from choline become the donor groups of AdoMet. Since PEMT probably represents the only endogenous source of choline, it would be interesting to observe the effects of its absence on choline metabolism. For example, do *Pempt* (-/-) mice suffer neurological damage from a lack of acetylcholine? In the liver, since PEMT is both a consumer and a producer of AdoMet, how does PEMT's absence affect the levels of this methyl group donor? Is the release of choline from PC inhibited in mice lacking PEMT, in order to conserve choline?

A related question is the ability of the choline analogs monomethylethanolamine (MMEth) and dimethylethanolamine (DMEth) to substitute for choline. Substitution of dietary choline with MMEth and DMEth would presumably generate PMME and PDME. In *Pempt* (-/-) mice, these novel phospholipids could not be converted to PC. Therefore, by comparing the effects on *Pempt* (-/-) mice of MMEth- and DMEth-supplemented diets compared to CS diets, the ability of PMME and PDME to replace PC would be tested, providing insights into the specificity of various liver functions such as lipoprotein and bile secretion for the choline head group of PC.

6.2.6 PEMT during fasting

Choline-deficiency generates deleterious effects in mice lacking PEMT, as described in Chapter 4. However, a choline-deficient diet is a laboratory-created condition; all natural diets contain choline, primarily as PC. Nonetheless, choline-deficiency does occur in the wild during starvation. Therefore, the effects of fasting on *Pempt* (-/-) mice could be observed and compared to the effects of a CD diet. There are important differences between fasting and a CD diet though. Fasting will reduce lipoprotein metabolism, because there is no lipid intake. Instead, lipids will be consumed by β-oxidation to provide energy. As well, bile secretion will be reduced. Therefore, the demand for PC may be lower in fasted mice compared to mice fed a CD diet, mitigating the effects of the choline-deficiency.

6.2.7 The progression and reversibility of liver damage in Pempt *(-/-) mice fed a CD diet*

The experiments in Chapter 4 were performed on mice fed a CD or CS diet for three days, at which point *Pempt* (-/-) mice appeared very unhealthy. However, the order of the appearance of the phenotypes (lower hepatic PC, fatty liver, bile accumulation in the gall bladder, elevated transaminases) is not known. By following these phenotypes at earlier time points, their relative order of appearance could be determined, allowing further insight into the mechanism of liver damage. As well, re-supplementation with choline at different time points would allow a determination of the reversibility of these phenotypes.

6.2.8 The consequences of choline-deficiency in non-hepatic tissues of Pempt *(-/-) mice*

As outlined in Chapter 4, choline-deficiency in mice lacking PEMT causes massive liver damage. However, the effects on other tissues have not been determined. Figure 3.5 demonstrates that choline derived from PEMT is distributed to non-hepatic tissues, therefore, it is not unreasonable to expect some effects there. Histological examination of these tissues and measurement of their PC levels would be two initial steps.

6.2.9 The role of PEMT as a regulator of hepatocyte growth

Based on its ability to inhibit hepatoma cell growth (15) and its absence from rapidly-dividing or tumourigenic hepatocytes (15-18), PEMT has been identified as a potential tumour suppressor. Therefore, it would be reasonable to expect a higher rate of hepatocellular carcinoma in *Pempt* (-/-) mice than in *Pempt* (+/+) or (+/-) mice. So far, this has not been observed, although the colony is less than a year old. Mice of all three genotypes will be allowed to age, and their relative rates of liver cancer determined. As well, the susceptibility of *Pempt* (-/-) to carcinogens will be measured and compared to that of *Pempt* (+/+) and (+/-) mice.

References

1. Sundler, R., and Akesson, B. (1975) *Journal of Biological Chemistry* **250**(9), 3359-3367
2. Scarborough, G. A., and Nyc, J. F. (1967) *Journal of Biological Chemistry* **242**(2), 238-242
3. Kodaki, T., and Yamashita, S. (1987) *Journal of Biological Chemistry* **262**(32), 15428-15435
4. Kodaki, T., and Yamashita, S. (1989) *European Journal of Biochemistry* **185**(2), 243-251
5. Preitschopf, W., Luckl, H., Summers, E., Henry, S. A., Paltauf, F., and Kohlwein, S. D. (1993) *Current Genetics* **23,** 95-101
6. Kanipes, M. I. (1997) thesis in *Department of Biological Sciences*, pp. 147, Carnegie Mellon University, Pittsburgh, Pennsylvania
7. Bansal, V. S., and Kanfer, J. N. (1985) *Biochemical & Biophysical Research Communications* **128**(1), 411-416
8. Zeisel, S. H., Epstein, M. F., and Wurtman, R. J. (1980) *Life Sciences* **26,** 1827-1831
9. Zeisel, S. H., and Wurtman, R. J. (1981) *Biochemical Journal* **198,** 565-570
10. Vance, J. E., Nguyen, T. M., and Vance, D. E. (1986) *Biochimica et Biophysica Acta* **875**(3), 501-509
11. Nishimaki-Mogami, T., Suzuki, K., and Takahashi, A. (1996) *Biochimica et Biophysica Acta* **1304**(1), 21-31
12. Vance, J. E., and Vance, D. E. (1986) *FEBS Letters* **204**(2), 243-246
13. Holub, B. J., and Kuksis, A. (1971) *Canadian Journal of Biochemistry* **49**(12), 1347-1356

14. Samborski, R. W., Ridgway, N. D., and Vance, D. E. (1993) *Journal of Lipid Research* **34**(1), 125-137

15. Cui, Z., Houweling, M., and Vance, D. E. (1994) *Journal of Biological Chemistry* **269**(40), 24531-24533

16. Tessitore, L., Cui, Z., and Vance, D. E. (1997) *Biochemical Journal* **322**(Pt 1), 151-154

17. Houweling, M., Cui, Z., Tessitore, L., and Vance, D. E. (1997) *Biochimica et Biophysica Acta* **1346**(1), 1-9

18. Cui, Z., Shen, Y. J., and Vance, D. E. (1997) *Biochimica et Biophysica Acta* **1346**(1), 10-16

Appendix 1

Does the liver-enriched nuclear factor C/EBPα stimulate *Pempt* transcription?

App. 1.1 Introduction

Phosphatidylcholine (PC) constitutes the predominant phospholipid in eukaryotic membranes. All mammalians cells synthesize PC via the CDP-choline pathway, in which choline is phosphorylated to phosphocholine by choline kinase, then converted to CDP-choline by CTP:phosphocholine cytidylyltransferase, and finally lipidated with the addition of diacylglycerol by cholinephosphotransferase (1). The second step in this pathway, catalyzed by the cytidylyltransferase, is rate-limiting (2).

A second pathway for the biosynthesis of PC, the methylation of phosphatidylethanolamine (PE), occurs in mammals to a significant degree only in the liver (3). The conversion of PE to PC is accomplished in three distinct steps, all of which are catalyzed by the enzyme phosphatidylethanolamine *N*-methyltransferase (PEMT). This enzyme has been purified to homogeneity, and the amino terminus partially sequenced (4). This peptide sequence was used to design oligonucleotides to screen a rat liver cDNA library (5). Clones were isolated, sequenced and used to design peptides to generate antibodies to the C-terminus of the encoded protein. Interestingly, subcellular fractionation and immunolocalization studies suggested two isoforms of PEMT: PEMT1 localized to the endoplasmic reticulum, and PEMT2, encoded by the cDNA and localized to the mitochondria-associated membrane (6). The mouse gene for PEMT2, named *Pempt*, was cloned using the rat PEMT2 cDNA as a probe (7). The gene is localized to chromosome 11, 31 centimorgans from the centromere, where its seven exons and six introns span at least 35 kb. The promoter region contains no identifiable TATA box. Recently, gene targeting has resulted in a mouse in which the *Pempt* gene has been disrupted (8). Mice carrying two copies of the

disrupted *Pempt* allele contain virtually no PEMT activity in their livers, suggesting that PEMT1 and PEMT2 are encoded by the same gene.

Several interesting aspects of *Pempt* gene expression form a basis for the study of its transcriptional regulation. Rat PEMT2 protein expression is detectable only in the liver, in concordance with the enzyme activity distribution (5). As well, there is a correlation between the growth rate of hepatocytes and *Pempt* expression. Specifically, when hepatocytes are dividing rapidly, *Pempt* expression is low or undetectable. However, when hepatocytes are fully differentiated and quiescent, *Pempt* expression is stimulated. This correlation has been demonstrated in several experimental systems. In the developing rat embryo, no *Pempt* expression is observed as hepatocytes proliferate (9). However, at birth, as hepatocytes undergo final differentiation, *Pempt* expression increases dramatically. When hepatocytes are chemically induced to proliferate, either tumourigenically, or through non-neoplastic cell division, *Pempt* expression is reduced (10). As well, when hepatocytes proliferate following partial hepatectomy, *Pempt* gene expression is reduced (11). Finally, extended choline-deficiency, which inhibits PC biosynthesis via the CDP-choline pathway, stimulates *Pempt* gene expression, presumably in order to maintain hepatic PC levels (12). These results suggest three bases for studying *Pempt* gene regulation: tissue-specificity, growth stage and dietary modulation.

The cloning of the cDNA and gene for PEMT has given us valuable tools for the study of *Pempt* gene regulation. The cDNA has already been found to be useful as a probe for detecting PEMT mRNA in RNA blots. The *Pempt* gene clones containing 5' upstream regions could be sequenced and analyzed for the presence of consensus binding sites of transcription activators. As well,

subclones of the 5' upstream region could be subcloned into reporter vectors, in order to analyze their importance in the transcriptional activation of the *Pempt* gene. Initial experiments of this kind are described in this chapter.

Unfortunately, another important tool for the study of *Pempt* gene regulation is unavailable. There is no immortalized cell culture line yet known that expresses PEMT. This fact is probably related to the correlation between cell growth and PEMT noted above: since immortalized culture cells divide rapidly, it is not surprising that they do not contain PEMT. This fact precludes an important early step in the study of any gene's regulation: the relationship between gene expression and a given treatment.

At the same time, the observation that no known cell line expresses PEMT in culture resulted in an important insight into a possible mechanism of *Pempt* gene regulation. The transcription factor C/EBPα (for CCAAT/enhancer binding protein), like PEMT, is not expressed in any known cell line (13). The embryonic expression pattern of C/EBPα closely matches that of PEMT, except that significant expression begins just prior to birth in the liver, instead of the actual day of birth (14). Like PEMT, C/EBPα levels are reduced in the regenerating liver (11,15). C/EBPα is enriched in the liver, as well as adipocytes, and to a lesser extent lung (14). Finally, C/EBPα has been implicated in the regulation of several genes involved in lipid metabolism, including those for acetyl CoA-carboxylase (16), apolipoprotein B (17) and the liver fatty acid-binding protein (18). These facts led to the hypothesis that the transcription factor C/EBPα plays an important role in the regulation of *Pempt* gene expression.

App. 1.2 Materials and Methods

App. 1.2.1 Isolation of primary rat hepatocytes

Hepatocytes were isolated from adult female rat livers by collagenase perfusion (19,20). An adult female rat was anaesthetized, then incised through the abdomen to expose the hepatic portal vein. The portal vein was canulated, and Hanks' Balanced Salt Solution (Gibco) without Ca^{2+} and Mg^{2+} pumped through the liver until blood was completely removed via an incision in the heart. The perfusate was replaced with Hanks' solution containing 6 mg/ml collagenase, and perfusion continued for 5 minutes. The liver was excised, mashed with scissors, and further digested in collagenase solution to completely separate cells. The cell suspension was filtered through a wire mesh to remove debris, then washed twice with Dubelcco's Modified Eagle's medium (DME) (Gibco), followed by a single wash with DME containing 17% delipidated fetal bovine serum (FBS) (Gibco). Individual cells were counted with a haemocytometer, and plated at 2 x 10^6 cells per 10-cm dish, generating approximately 30 dishes from a single liver. Cells were cultured in DME containing 17% FBS, and allowed to attach for 2 h. Cell culture continued at 37°C, 5% CO_2 for several days.

App. 1.2.2 Preparation and analysis of RNA

PolyA-enriched RNA was isolated from cultured hepatocytes and McArdle RH7777 cells, using the QuickPrep Micro mRNA Purification kit purchased from Pharmacia Biotech, and following the manufacturer's instructions. RNA isolated from a single plate was electrophoresed and blotted

onto nylon according to standard techniques (21). The RNA blot was probed with rat PEMT2 cDNA, radiolabeled by random priming. Hybridization conditions were identical to those used for DNA blotting (Chapter 2) (7). The RNA blot was visualized by phosphorimaging. In order to assure equal loading of RNA samples, the RNA blot was stripped in boiling 0.25% SDS, then re-probed with human β-actin cDNA under the same conditions.

App. 1.2.3 Sequencing of the 5' upstream region of the Pempt *gene*

A subclone of clone 2614 (called 14/H3/5) containing exon 1 of the *Pempt* gene and the adjacent 5' region was purified using a Wizard Miniprep kit (Promega), and sequenced by the DNA Core facility on an automated sequencer (Applied Biosystems Inc./Perkin-Elmer model 373a). Sequencing primers (Fig. App. 1.1) were based on previously-sequenced regions of 14/H3/5. Both strands of the DNA were sequenced to ensure accuracy. Potential C/EBPα binding sites were identified by similarity to published consensus binding sites, including GTGG$^{T}/_{A}$$^{T}/_{A}$$^{T}/_{A}$G, ATTGC (22), $^{G}/_{A}$TTGCG$^{C}/_{T}$AA$^{C}/_{T}$ (23), T$^{T}/_{G}$NNG$^{C}/_{T}$AA$^{T}/_{G}$ (24) and T$^{T}/_{G}$NNGNAA$^{T}/_{G}$ (25).

App.1.2.4 Construction of reporter vectors

Fragments of the 5' upstream region of the *Pempt* gene were amplified by the polymerase chain reaction (PCR), using subclone 14/H3/5 as a template and the oligonucleotides indicated in Figure App. 1.1. The PCR fragments were cloned in the vector pCR 2.1, using the TA Cloning kit (Invitrogen) and following the manufacturer's instructions. The fidelity of amplification was checked by sequencing the cloned PCR products. The cloned PCR fragments containing

sections of the 5' region of the *Pempt* gene were subcloned into the vector pGL3 Basic (Promega) which contains a modified firefly luciferase cDNA. Large amounts of the reporter vectors were purified from 1L cultures of transformed *Eschrichia coli* using the Wizard Maxiprep kit (Promega) according to the manufacturer's instructions, followed by CsCl gradient centrifugation. DNA concentration was measured fluorometrically (26), using salmon sperm DNA as a standard.

App. 1.2.5 Maintenance and transfection of cultured cells

The rat hepatoma cell line McArdle RH7777 was maintained as monolayer cultures in uncoated 10-cm dishes, under DME containing 10% FBS and 10% horse serum. Incubation was at 37°C in 5% CO_2.

Twenty-four h prior to transfection, cells were plated at ~30% confluence in 3-cm 6-well dishes. Transfection mix was prepared by combining for each well 4μg C/EBPα expression plasmid DNA (gift of Dr. B. M. Spiegelman), with either pSV-SPORT (SV40 early promoter) or pCI (cytomegalovirus promoter) as the vector backbone, or 4μg pCI expression vector DNA, 4 μg of reporter vector DNA and 1 μg β-galactosidase expression plasmid (gift of Dr. L. B. Agellon). Transfection was mediated by liposomes made with 45 μg per well of *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethyl-ammonium methylsulfate (DOTAP), and following published protocols (27). Cells were harvested 40 hs after transfection, using Reporter Lysate Buffer (Promega) and following the manufacturer's instructions.

App. 1.2.6 Enzyme and protein detection and measurement

Total protein mass in the lysates was measured using the BCA assay kit (Pierce) with bovine serum albumin as a standard. In order to detect expression of C/EBPα, 50 μg of protein per lane was electrophoresed in 12% polyacrylamide with 0.1% SDS, then transferred onto Immobilon-P membranes (Millipore) and probed with anti-rat C/EBPα antibody (Santa Cruz Biotechnology, Inc.). Molecular masses were determined by comparison to known standards.

Luciferase activity in 15 μL of lysate was measured using the Luciferase Assay System (Promega), with a Lumat Model #LB 9501 luminometer. β-Galactosidase activity in 40 μL of lysate was measured with the β-Galactosidase Assay System (Promega), with a SpectraMax 250 microtiter plate reader (Molecular Devices). In order to correct for variation in transfection efficiency, luciferase activity was normalized to β-galactosidase activity for each well.

App. 1.3 Results

App. 1.3.1 Pempt *gene expression in primary hepatocytes*

We were interested in determining whether a primary culture of rat hepatocytes, unlike immortalized cell lines, expressed the *Pempt* gene. Previous experiments have demonstrated the presence of PEMT activity in primary rat hepatocytes (28-30). Hepatocytes were isolated and plated. Initially, the hepatocytes maintained the polyhedral morphology seen in the liver. However, during the course of several days, the cells took on a more fibroblastic

appearance. Using rat PEMT2 cDNA as a probe, PEMT mRNA was detected in RNA isolated from primary cultures 2 h after isolation (Fig. App. 1.2). However, 50 h after isolation, no PEMT2 mRNA was detected in the primary hepatocyte culture. Therefore, it appears that rat Pempt gene expression is inhibited by alteration of hepatic tissue organization. We assume that the decrease in expression is due at least partly to an inhibition of transcription. It is interesting to note that C/EBPα expression is also inhibited by the process of hepatocyte isolation (15,31), a further demonstration of the concordance of C/EBPα and PEMT expression. Because PEMT2 mRNA levels are reduced upon isolation, we do not believe that primary cultures of hepatocytes offer any advantages over immortalized cell lines for the study of *Pempt* gene expression, particularly for transient transfection experiments that require several days of culture time.

App. 1.3.2 Cotransfection of C/EBPα and Pempt *promoter reporter constructs*

In order to determine whether C/EBPα influences *Pempt* gene transcription, we took advantage of a technique that has previously been employed to examine the effect of C/EBPα on the transcription of the genes for acetyl-CoA carboxylase (16), albumin (13), glutathione *S*-transferase (32) and alcohol dehydrogenase (33). We constructed three vectors containing various portions of the 5' upstream region linked to a promoterless luciferase reporter gene: -37 to +127, -607 to +127 and -962 to +127 (+1 is the first transcription start site) (Fig. 5.3A). Sequence analysis of this DNA revealed several potential C/EBPα binding sites (Fig. App. 1.1). These reporter vectors were transfected via liposomes into McArdle RH7777 rat hepatoma cells, along with a C/EBPα expression vector or the expression vector without the C/EBPα insert. A β-

galactosidase expression vector was also transfected into the cells in order to normalize the different samples for transfection efficiency. Forty h after transfection, the cells were harvested, and assayed for luciferase activity and β-galactosidase activity. Compared to the promoterless luciferase vector, all three vectors containing 5' upstream regions of the *Pempt* gene had only approximately 20-fold higher luciferase activity, while the positive control plasmid (luciferase under the control of SV40 promoter) had 268-fold higher luciferase activity (Fig. App. 1.3B). This result suggests that in the absence of C/EBPα, *Pempt* is only weakly transcribed from its own promoter. However, when the reporter vectors were co-transfected with C/EBPα expression vector, luciferase activity was significantly stimulated. For the reporter plasmid -37 to +127, luciferase activity was stimulated 18.1-fold compared to when C/EBPα is absent, and 378-fold compared to promoterless luciferase. Similar changes were observed for the -603 to +127 and -962 to +127 plasmids. In contrast, only a two-fold stimulation of luciferase activity was observed in the promoterless luciferase and SV-40 luciferase vectors upon co-transfection with the C/EBPα expression plasmid. These results suggest that C/EBPα is a specific *trans* activator of *Pempt* transcription, and that this activation requires only the DNA segment -37 to +127 of the gene.

As shown in figure App. 1.1, the region -37 bp +127 of the *Pempt* gene does not contain an obvious C/EBPα binding site based on homology to other genes regulated by this transcription factor. Nonetheless, a reporter construct containing a mutation within this region was generated. Specifically, a four nucleotide substitution between -37 and -30 was performed using PCR. This construct was transfected in McArdle RH7777 cells, with and without C/EBPα co-transfection. Without C/EBPα co-transfection, the mutant vector generated

only 28% of the luciferase activity of its wild-type counterpart, suggesting that the mutation affected basal promoter activity (Fig. App. 1.4). With C/EBPα co-transfection, this effect was moderated: the mutant construct generated 63% of the activity of the wild-type construct. Therefore, the addition of C/EBPα-encoding plasmid resulted in a 112-fold stimulation of transcriptional activity from the mutant reporter plasmid, compared to a 50-fold stimulation of transcriptional activity from the wild-type plasmid. Therefore, it appears that the four base substitution did not significantly alter C/EBPα transactivation of the *Pempt* promoter. It is worthwhile to note that C/EBPα co-transfection stimulated transcription from the wild-type -37 to +127 construct 50-fold in this experiment, compared to 378-fold for the previous experiment (Figs. App. 1.3B and App. 1.4). As well, for the promoterless luciferase vector pGL3 Basic, the increase in transcription caused by C/EBPα co-transfection varied from two-fold to 50-fold. These results demonstrate the variability of these experiments, for which no obvious explanation is available.

App. 1.3.3 Immunoblot of proteins from C/EBPα -transfected cells

In order to ensure that C/EBPα was properly expressed in McArdle RH7777 cells following transfection, lysates from transfected cells were subjected to immunoblot analysis, using an antibody raised against rat C/EBPα. As expected, in cells transfected with empty expression plasmid, no band corresponding to 42 kDa C/EBPα was detected (Fig. App. 1.5). Surprisingly, no immunoreactive band was detected in C/EBPα-transfected cells corresponding to the native 42 kDa C/EBPα protein either. Instead, two bands, one corresponding to approximately 50 kDa and one corresponding to approximately 30 kDa were observed. The intensity of the 50 kDa band was

elevated in cells transfected with the C/EBPα-containing expression plasmid compared to cells transfected with the empty expression plasmid. Thus it appears that McArdle RH7777 cells generate a form of C/EBPα different from the native form. This result casts doubt on the relevance of the results described above to what actually occurs in the liver, since they appear to be due to the effects of a non-native form of C/EBPα.

App. 1.4 Discussion

Little is known about the mechanisms regulating the expression of phospholipid biosynthesis genes in mammals. This situation contrasts greatly with that in yeast, where the transcriptional control mechanisms of these genes, including those encoding PEMT, have been studied extensively. The yeast PEMT genes, *PEM1/CHO2* and *PEM2/OPI3* are members of a group of phospholipid biosynthesis genes whose transcription is repressed in the presence of exogenous inositol and choline (34). These genes have in common a 10 -base pair sequence (5'-CATGTGAAAT-3') designated as the inositol sensitive upstream activating sequence, or UAS_{INO} (35-39). A version of the UAS_{INO} consensus sequence, 5'-CATRTGAA-3', was identified in the upstream region of both the *PEM1* and *PEM2* genes (37). Two inositol sensitive gene products, INO2 and INO4, bind to this element (39-42). These genes encode proteins containing the basic helix-loop-helix (bHLH) motif (43,44), while the UAS_{INO} contains the canonical bHLH binding site (35,39,41). Henry and Patton-Vogt have proposed a model whereby phosphatidic acid, or a metabolite thereof, is the initial metabolic signal responsible for the eventual activation of the phospholipid biosynthesis genes via activation of INO2 and INO4 (34).

However, the regulation of PEMT in mammals is most likely different from that in yeast. Evidence suggests that *Pempt* expression is strongly and inversely correlated with the growth rate of hepatocytes: rapidly-dividing hepatocytes, such as during embryogenesis or tumorigenesis, do not express PEMT (5,9-11). However, fully-differentiated, non-dividing hepatocytes do express PEMT. This situation is the reverse of that in yeast, where *PEM1* and *PEM2* are maximally expressed during the logarithmic growth phase (45,46). This fact, plus the additional issues of tissue specificity and that the *PEM* version of the UAS_{INO} sequence is not present in the 5'upstream region of the *Pempt* gene, suggests that yeast PEMT is regulated very differently from mammalian PEMT.

One advantage for the study of a gene's regulation is a cell culture line that expresses the gene endogenously. No such cell line has been found yet for *Pempt*. Liver-derived cell lines such as McArdle RH7777 are typically isolated from hepatomas, and are thus rapidly-dividing, precluding *Pempt* expression. Figure 5.2 demonstrates that primary hepatocytes also lose the ability to express *Pempt*. This downregulation of expression has been previously observed for other liver-specific genes, including albumin, major urinary protein, α1-antitrypsin, transthyretin and as mentioned above, C/EBPα (15,31,47,48). Nuclear run-on assays have implicated transcription inhibition as the cause of the change in expression. Therefore, isolated primary hepatocytes are not more useful than immortalized cell lines for the study of *Pempt* expression. Recent advances have been made in the generation of cell lines from liver that express liver-specific genes (49,50), that take advantage of the serum-free, hormonally defined media that has been found to prolong the liver-specific functions of isolated hepatocytes (51). It would be worthwhile to determine if

PEMT is expressed in these cell lines, so they could be used to study the effects of various treatments on gene expression.

The expression pattern of the transcription factor C/EBPα closely matches that of PEMT, i. e. it is expressed only fully-differentiated, non-proliferating hepatocytes. As well, the immediate 5' upstream region of the *Pempt* gene contains putative binding sites for C/EBPα. Therefore, we investigated whether C/EBPα could stimulate *Pempt* transcription, using reporter constructs containing different lengths of the 5' upstream region. A construct containing as little as 164 base pairs of the proximal promoter, from -37 to +127, was transcriptionally stimulated by C/EBPα in McArdle RH7777 cells. However, this region contains no obvious C/EBPα binding site, based on homology to previously published sequence. It is worth nothing though that C/EBPα is rather promiscuous in its DNA binding specificity (22). Therefore, DNA footprinting experiments should be used to precisely identify and define the C/EBPα binding site. Reporter constructs containing wild-type and mutated versions of this binding site can then be tested for their ability to activate transcription, thus demonstrating the importance of C/EBPα for *Pempt* expression.

McArdle RH7777 cells transfected with a C/EBPα expression vector do not express the wild-type version of the protein, although partial sequencing of the plasmid does not suggest any mutations. The 50 kDa protein observed in these cells is not seen in HepG2 cells (a human hepatoma cell line), which express wild-type C/EBPα upon transfection (13). Therefore, the origin of this 50 kDa is likely some post-translational modification that does not occur in other cell lines. The 30 kDa band is most likely due to the use of an alternate

translation start site, as has been previously demonstrated (52-54). Therefore, further experiments should be carried out in other cell lines that express the native form of C/EBPα following transfection, such as HepG2. As an (expensive) alternative, transgenic mice carrying various reporter constructs can be generated. Such mice have the additional benefit of revealing any relationship between the 5' upstream region and tissue-specificity.

Figure App.1.1 **Sequence of the proximal promoter region of the mouse *Pempt* gene.**

Underlined sequences represent potential C/EBPα binding sites. Asterices denote transcription start sites. +1 is the first transcription start site. Oligonucleotides used for sequencing and PCR are shown above the sequence. Changes in the mutant -37 to +127 plasmid are shown below the sequence.

-962 Sense >

GGTCAGTCAAGGCACTGTCCACCTATCTGAAAAGGTGAGAGAAGGCACAACTGCAGCTTCC

-947

CTCTGATTGTTACTAGGGATGAGCCTAGAACCCTCAAACGTGCTAGGTAAGCGCTCTATCA

-857

CTGAGCCATGGCTGACAGCCTGAAACTTGTATTTATTTTTTTACTGAATGTTGCTGGGTCA

GTGTCTTCTGGTTCTTGCTTTAGGATACATGCCTGTCGAGAGAGTGAGAGGTGACACATCT

GTATTTCTTAGTTAGGATGTAGCAGAAGAAAGTCCAGGTGGCTTGACACCAGGGCCTTCAC

-681

< -610 Antisense -607 Sense

ATCTGTATCTGACATCTGCTCTTCACTCTGTTCATGGGTCATCTTGGGTGGGTGGGCATTC

>

TTGTTAACCCACTTCGGTTAAGGAACCTGACCTCAGATCAGTCCAGGTGGTTACTGTGAGC

-550

TCTGTGGGCATTCAGGAGGAGGGTAGAGCTCTGTCCTTCACTGGGTCCTTCCTTAGATCCA

GGGAGGGCACAGTGTATGAAATGGGGCAGAAAGGATGCCTTAATATGGTAAACATGGTGCC

TCCCCCTCTTGGGAGTGTAGACCTAGGTAGAGAGGTTCCAGTACCTTGCTTCTTTCTTTCA

< -308 Antisense

GGCCACAGGGCTCTTGTGGAATTGCCCAGCGAATGTTCCTGGATCTGCTGGTAATTCCTGG

-335

ACACTGGGATAGCTGGGAAGAGGGCGAAGAGTATGCAATTGCAGAGGGCCACTGTATGGGA

-254

TGAAGGAACCTGTCCCCGGTCTGAGACAGCCCCACTCTGTCCCTTTTATCCTTTGATGTGT

ATGGTACATGTCACAGAGAGGCCCATGACTTTCAGGTGATCAATGCAACCCTGGCTGTCTG

GCACTCTGGCTGTTTGTTTGGATGTCACAATGCAGCTTGCACCCTGGGTGCTGTTGACCCC

-37 Sense > * *

CTGCAAATCATTCCCTAACTACAGAACATTGCTCTGAAGTGTGTCCAGGTGCCCAGTGTGG

A A GG

TAGAACTGCTATGTGCCGCCTTCCTTTCAGCTTGTGGGGGTTCTGCAGCTGAGTTCATCAT

< +127 Antisense

CAGGGTGACCTGAGCTGTCCCTGGAGGCTGGCTCCCATTTCCTTCTGGTTCTG

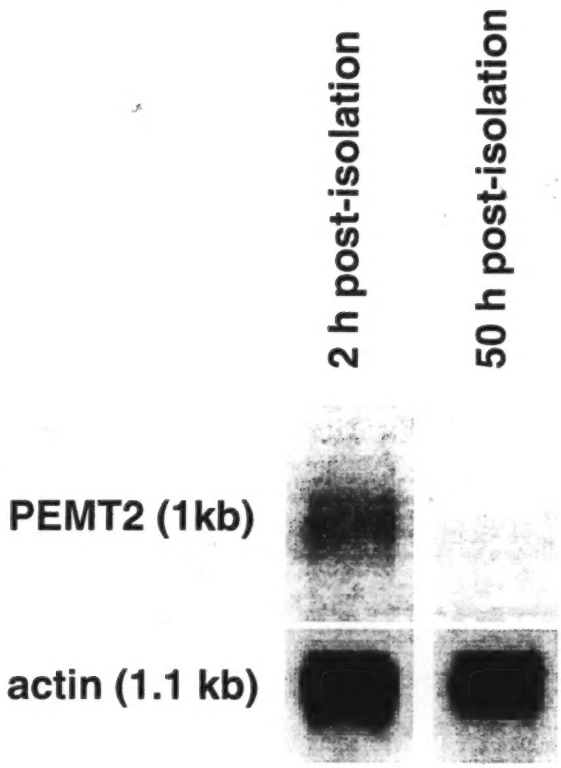


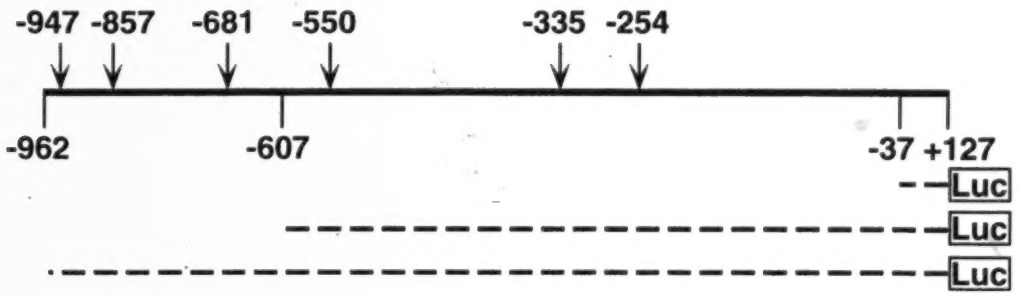
Figure App. 1.2 ***Pempt*** **gene expression is reduced in isolated hepatocytes.**

Hepatocytes were isolated from a rat and plated. Two h and fifty h following isolation, mRNA was isolated from the hepatocytes and tested for the presence of PEMT2 mRNA, using a cDNA for rat PEMT2 as a probe on RNA blots. RNA blots were also probed for actin, to ensure equal loading of intact mRNA.

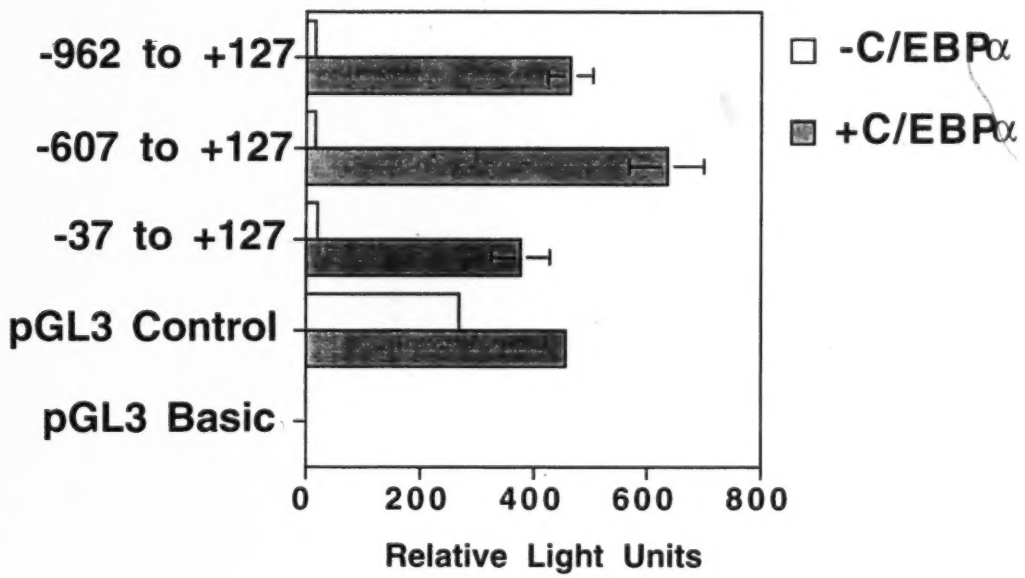
Figure App.1.3 **C/EBPα stimulates transcription from the *Pempt* promoter.**

(A) Schematic of the *Pempt* 5'-upstream region. Potential C/EBPα binding sites are marked by arrows (see Fig. 2). Below are shown the various reporter constructs. (B) Cells were transfected with reporter constructs, and expression plasmid either with (+C/EBPα) or without (-C/EBPα) a C/EBPα-encoding insert. Forty h after transfection, cells were harvested and assayed for luciferase activity. Activity is expressed in light units relative to that generated with a promoterless luciferase plasmid (pGL3 Basic) without C/EBPα. Transfection efficiency was normalized by co-transfection with a β-galactosidase plasmid, and assaying for β-galactosidase activity. As a positive, cells were transfected with a vector encoding luciferase under the control of the SV-40 viral promoter (pGL3 Control). Error bars represent standard deviations (n=3).

A.


-947 -857 -681 -550 -335 -254
-962 -607 -37 +127
Luc
Luc
Luc

B.


-962 to +127
-607 to +127
-37 to +127
pGL3 Control
pGL3 Basic
-C/EBPα
+C/EBPα
0 200 400 600 800
Relative Light Units

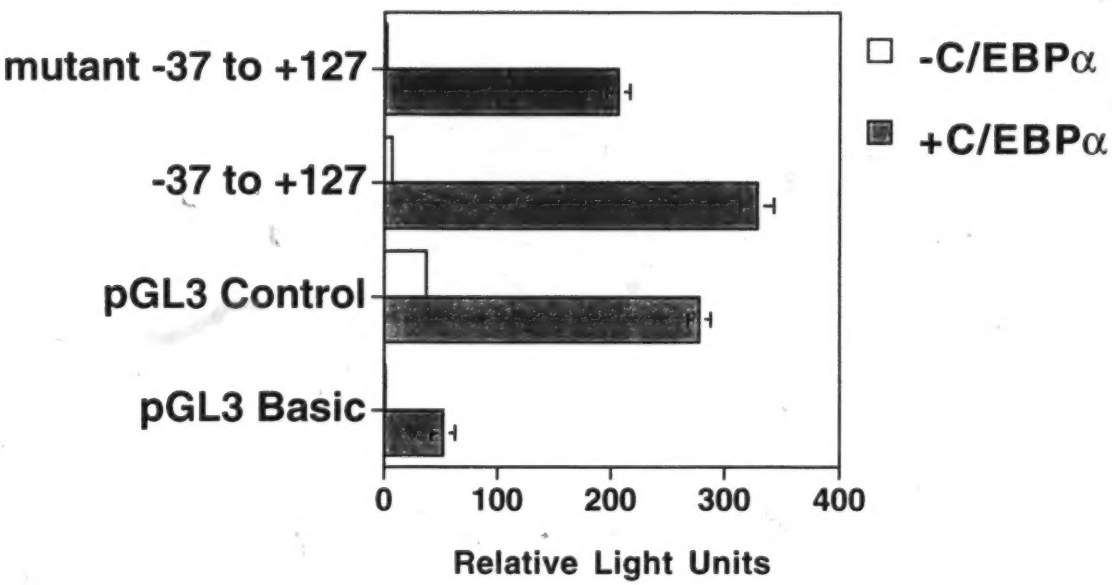


Figure App. 1.4 **Mutation of -37 to +127 region of the *Pempt* gene does not affect C/EBPα-dependent induction of transcription.**

The reporter construct -37 to +127 was mutated at its 5' end by the use of a mismatched oligonucleotide for PCR. This construct was transfected into McArdle RH7777 cells and assayed for luciferase as before (Fig. App. 1.3). Error bars represent standard deviations (n=3).

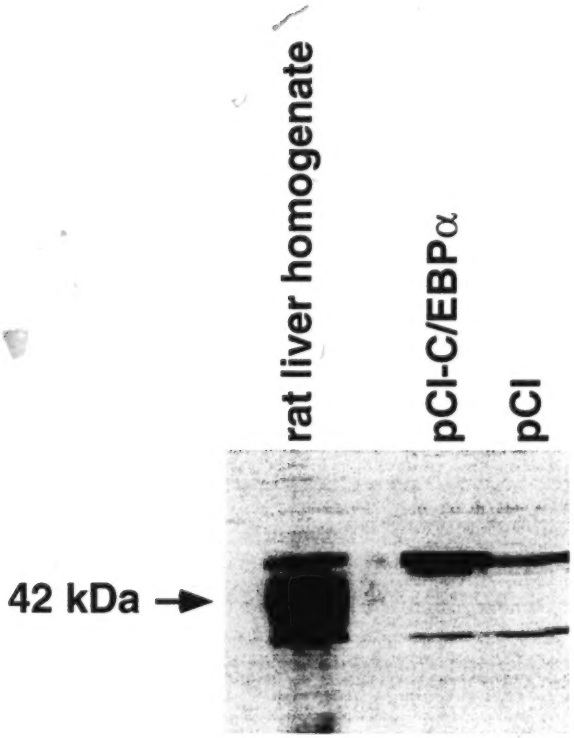


Figure App. 1.5 **McArdle RH7777 cells transfected with a C/EBPα expression plasmid do not express the native form of this protein.**

Cells were transfected with an expression plasmid with (pCI-C/EBPα) or without (pCI) a C/EBPα insert. 40 hs after transfection, cells were harvested, and C/EBPα protein detected by immunoblotting. Positive control is rat liver homogenate. 50 μg protein per lane.

References

1. Kennedy, E. (1989) in *Phosphatidylcholine Metabolism* (Vance, D. E., ed), pp. 1-8, CRC Press, Boca Raton
2. Vance, D. E., Trip, E. M., and Paddon, H. B. (1980) *Journal of Biological Chemistry* **255**(3), 1064-1069
3. Ridgway, N. D. (1989) in *Phosphatidylcholine metabolism* (Vance, D. E., ed), pp. 103-120, CRC Press, Boca Raton
4. Ridgway, N. D., and Vance, D. E. (1987) *Journal of Biological Chemistry* **262**(35), 17231-17239
5. Cui, Z., Vance, J. E., Chen, M. H., Voelker, D. R., and Vance, D. E. (1993) *Journal of Biological Chemistry* **268**(22), 16655-16663
6. Vance, J. E. (1990) *Journal of Biological Chemistry* **265**(13), 7248-7256
7. Walkey, C. J., Cui, Z., Agellon, L. B., and Vance, D. E. (1996) *Journal of Lipid Research* **37**(11), 2341-2350
8. Walkey, C. J., Donohue, L. R., Bronson, R., Agellon, L. B., and Vance, D. E. (1997) *Proceedings of the National Academy of Science, USA* **in press**
9. Cui, Z., Shen, Y. J., and Vance, D. E. (1997) *Biochimica et Biophysica Acta* **1346**(1), 10-16
10. Tessitore, L., Cui, Z., and Vance, D. E. (1997) *Biochemical Journal* **322**(Pt 1), 151-154
11. Houweling, M., Cui, Z., Tessitore, L., and Vance, D. E. (1997) *Biochimica et Biophysica Acta* **1346**(1), 1-9
12. Cui, Z., and Vance, D. E. (1996) *Journal of Biological Chemistry* **271**(5), 2839-2843
13. Friedman, A. D., Landschulz, W. H., and McKnight, S. L. (1989) *Genes & Development* **3**, 1314-1322

14. Birkenmeier, E. H., Gwynn, B., Howard, S., Jerry, J., Gordon, J. I., Landschulz, W. H., and McKnight, S. L. (1989) *Genes & Development* **3,** 1146-1156

15. Mischoulon, D., Rana, B., Bucher, N. L. R., and Farmer, S. R. (1992) *Molecular and Cellular Biology* **12**(6), 2553-2560

16. Tae, H.-J., Luo, X., and Kim, K.-H. (1994) *Journal of Biological Chemistry* **269**(14), 10475-10484

17. Kardassis, D., Zannis, V. I., and Cladaras, C. (1992) *Journal of Biological Chemistry* **267**(4), 2622-2632

18. Simon, T. C., Roth, K. A., and Gordon, J. I. (1993) *Journal of Biological Chemistry* **268**(24), 18345-18358

19. Davis, R. A., Engelhorn, S. C., Pangburn, S. H., Weinstein, D. B., and Steinberg, D. (1979) *Journal of Biological Chemistry* **254,** 2010-2016

20. Vance, D. E., Weinstein, D. B., and Steinberg, D. (1984) *Biochimica et Biophysica Acta* **792**(1), 39-47

21. Kingston, R. E. (1987) in *Current Protocols in Molecular Biology*, pp. 4.1.1 -4.10.8, John Wiley & Sons, New York

22. Faisst, S., and Meyer, S. (1992) *Nucleic Acids Research* **20**(1), 3-26

23. Osada, S., Yamamoto, H., Nishihara, T., and Imagawa, M. (1996) *Journal of Biologocal Chemistry* **271**(7), 3891-3896

24. Ryden, T. H., and Beemon, K. (1989) *Molecular and Cellular Biology* **9,** 1155-1164

25. Akira, S., Isshiki, H., Sugita, T., Tanabe, O., Kinoshita, S., Nishino, Y., Nakajima, T., Hirano, T., and Kishimoto, T. (1990) *EMBO Journal* **9,** 1897-1906

26. Davis, L. G., Kuehl, W. M., and Battey, J. F. (1994) in *Basic Methods in Molecular Biology*, pp. 22-24, Appleton & Lange, Norwalk, CT

27. Holmen, S. L., Vanbrocklin, M. W., Eversole, R. R., Stapleton, S. R., and Ginsberg, L. C. (1995) *In Vitro Cell and Developmental Biology* **30,** 347-351

28. Vance, J. E., Nguyen, T. M., and Vance, D. E. (1986) *Biochimica et Biophysica Acta* **875**(3), 501-509

29. Vance, J. E. (1989) *Biochimica et Biophysica Acta* **1006**(1), 59-69

30. Ridgway, N. D., Yao, Z., and Vance, D. E. (1989) *Journal of Biological Chemistry* **264**(2), 1203-1207

31. Xanthopoulos, K. G., Mirkovitch, J., Decker, T., Kuo, C. F., and Darnell, J. E. (1989) *Proceedings of the National Academy of Science, USA* **86,** 4117-4121

32. Osada, S., Takano, K., Nishihara, T., Suzuki, T., Muramatsu, M., and Imagawa, M. (1995) *Journal of Biological Chemistry* **270**(52), 31288-31293

33. Stewart, M. J., Shean, M. L., and Duester, G. (1990) *Molecular and Cellular Biology* **10**(9), 5007-5010

34. Henry, S. A., and Patton-Vogt, J. L. (1997) *Progress in Nucleic Acids Research and Molecular Biology* , **in press**

35. Paltauf, F., Kohlwein, S., and Henry, S. A. (1992) in *The Molecular and Cellular Biology of the Yeast Saccharomyces* (Broach, J., Jones, E., and Pringle, J., eds), pp. 415-500, Cold Spring Harbor Laboratory Press, Plainview, NY

36. Lopes, J. M., Hirsch, J. P., Chorgo, P. A., Schulze, K. L., and Henry, S. A. (1991) *Nucleic Acids Research* **19,** 1687-1693

37. Kodaki, T., Hosaka, K., Nikawa, J., and Yamashita, S. (1991) *Journal of Biochemistry* **109**(2), 276-287

38. Kodaki, T., Nikawa, J., Hosaka, K., and Yamashita, S. (1991) *Journal of Bacteriology* **173**(24), 7992-7995

39. Ambroziak, J., and Henry, S. A. (1994) *Journal of Biological Chemistry* **269,** 15344-15349

40. Lopes, J. M., and Henry, S. A. (1991) *Nucleic Acids Research* **19,** 3987-3994

41. Nikoloff, D. M., and Henry, S. A. (1994) *Journal of Biological Chemistry* **269,** 7402-7411

42. Schwank, S., Ebbert, R., Rautenstrauss, K., Schweizer, E., and Schuller, H.-J. (1995) *Nucleic Acids Research* **23,** 230-237

43. Hoffman, D. R., Cornatzer, W. E., and Duerre, J. A. (1979) *Canadian Journal of Biochemistry* **57**(1), 56-65

44. Nikoloff, D. M., McGraw, P., and Henry, S. A. (1992) *Nucleic Acids Research* **20,** 3253

45. Homann, M. J., Poole, M. A., Gaynor, P. M., Ho, C. T., and Carman, G. M. (1987) *Journal of Bacteriology* **169**(2), 533-539

46. Lamping, E., Paltauf, F., Henry, S. A., and Kohlwein, S. D. (1995) *Genetics* **137,** 55-65

47. Clayton, D. F., and Darnell, J. E. J. (1983) *Molecular and Cellular Biology* **3,** 1552-1561

48. Clayton, D. F., Harrelson, A. L., and Darnell, J. E. J. (1985) *Molecular and Cellular Biology* **5**(10), 2623-2632

49. Zaret, K. S., DiPersio, C. M., Jackson, D. A., Montigny, W. J., and Weinstat, D. L. (1988) *Proceedings of the National Academy of Science, USA* **85,** 9076-9080

50. Wu, J. C., Merlino, G., and Fausto, N. (1994) *Proceedings of the National Academy of Science, USA* **91,** 674-678

51. Enat, R., Jefferson, D. M., Ruiz-Opaso, N., Gatmaitan, Z., Leinwand, L. A., and Reid, L. M. (1984) *Proceedings of the National Academy of Science, USA* **81,** 1411-1415

52. Descombes, P., and Schibler, U. (1991) *Cell* **67,** 569-579

53. Ossipow, V., Descombes, P., and Schibler, U. (1993) *Proceedings of the National Academy of Science, USA* **90,** 8219-8223

54. Lin, F.-T., MacDougald, O. A., Diehl, A. M., and Lane, M. D. (1993) *Proceedings of the National Academy of Science, USA* **90,** 9606-9610